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(54) Title: MUTANT ERα AND TEST SYSTEMS FOR TRANSACTIVATION

(57) Abstract: The present invention provides in general an artificial cell, an isolated mutant ERα, an isolated polynucleotide encoding the mutant ERα, a method for quantitatively analyzing an activity for transactivation of a reporter gene by a test ERα, a method for screening a mutant ligand dependent transcriptional factor, a method for evaluating an activity for transactivation of a reporter gene by a test ERα, a method for screening a compound useful for treating a disorder of a mutant ERα, a pharmaceutical agent useful for treating an estrogenic disorder of a mutant ERα, use of the mutant ERα, a method for diagnosing a genotype of a polynucleotide encoding a test ERα, a method for diagnosing a genotype of a polynucleotide encoding a test ERα and a method for diagnosing a phenotype of a test ERα.

DESCRIPTION

MUTANT ERα AND TEST SYSTEMS FOR TRANSACTIVATION

1. TECHNICAL FIELD OF THE INVENTION

The present invention relates to ligand dependent transcriptional factors, such as an ERα, and to genes encoding a ligand dependent transcriptional factor. Further, the present invention relates to cells containing a ligand dependent transcriptional factor and a specified reporter gene for the ligand dependent transcriptional factor.

2. BACKGROUND OF THE INVENTION

Various cell mechanisms are regulated by ligand dependent transcriptional factors. The regulation by the ligand dependent transcriptional factors is usually achieved because the ligand dependent transcriptional factor has an activity for transactivation of a gene. It has been postulated that in transactivation, the ligand dependent transcription factor and a RNA polymerase II complex interact together at a gene to increase the rate of gene expression. The transactivation can often determine in eukaryotic cells, whether a gene is sufficiently expressed to regulate the various cell mechanisms.

Such transactivation by the ligand dependent transcritpitional factors can occur when the ligand dependent tranactivational factor is selectively bound to its cognate ligand and to its cognate responsive element sequence. In this regard, the presence of the cognate responsive element in a gene or the presence of its cognate ligand in the cell can determine whether the ligand dependent transcriptional factor can transactivate the gene.

ERα is an example of such ligand dependent transcriptional factors. ERα is

naturally found in the target cells of estrogen such as in ovary cells, breast cells, uterus cells, bone cells and the like. The transactivation activity of ERα typically occurs when ERα is selectively bound to an ERE and an estrogen such as E2. It is reported that aberrant transactivation by ERα may contribute to various disorders. Attempts have been made to use anti-estrogens that are antagonistic to a normal ERα. Examples of such anti-estrogens used with such disorders include tamoxifen, raloxifene, 4-hydroxytamoxifen and the like.

3. SUMMARY OF THE INVENTION

The present invention provides in general an artificial cell, an isolated mutant ERα, an isolated polynucleotide encoding the mutant ERα, a method for quantitatively analyzing an activity for transactivation of a reporter gene by a test ERα, a method for screening a mutant ligand dependent transcriptional factor, a method for evaluating an activity for transactivation of a reporter gene by a test ERα, a method for screening a compound useful for treating a disorder of a mutant ERα, a pharmaceutical agent useful for treating an estrogenic disorder of a mutant ERα, use of the mutant ERα, a method for diagnosing a genotype of a polynucleotide encoding a test ERα, a method for diagnosing a genotype of a polynucleotide encoding a test ERα and a method for diagnosing a phenotype of a test ERα.

4. DESCRIPTION OF FIGURES

Figures 1 to 32 illustrate the luciferase activity provided by a human mutant ERα or a human normal ERα. The reporter gene was expressed in the chromosomes of the cell. The mutant ERα gene was transiently expressed in the cell. Figures 1, 2, 4, 5, 7, 8, 12, 13, 16, 17 and 21 to 26 illustrate the luciferase activity in the presence of various concentrations of 4-hydroxytamoxifen, raloxifene or ZM189154 as the sole probable

agent of stimulating a human mutant ERα or a human mutant ERα. Figures 3, 6, 9 to 11, 14, 15, 18 to 20, 27 to 32 illustrate the luciferase activity in the presence of various concentrations of 100pM of E2 with various concentrations of 4-hydroxytamoxifen, raloxifene or ZM189154. A stable transformed cassette cell was utilized to transiently express the human mutant ERα gene or the human normal ERα gene as well as to express in a chromosome thereof the reporter gene. Figures 1, 2, 4, 5, 7, 8, 12, 13, 16, 17 and 21 to 26 zero together the luciferase activity by using the luciferase activity provided by the controls, in which the human mutant ERα or the human normal ERα was in the presence of DMSO (containing no 4-hydroxytamoxifen, raloxifene or ZM189154). Figures 3, 6, 9-11, 14, 15, 18 to 20, 27 to 32 zero together the luciferase activity by using the luciferase activity provided by the controls, in which the human mutant ERα or the human mutant ERα was in the presence of a DMSO solution containing 100pM of E2. In zeroing the luciferase activity provided by the human mutant ERα and human normal ERα, the luciferase activity by the controls were set together as 100% luciferase activity. The luciferase activity provided by the controls in Figures 1, 2, 4, 5, 7, 8, 12, 13, 16, 17 and 21 to 26 is shown as DMSO. The luciferase activity provided by the controls in Figures 3, 6, 9-11, 14, 15, 18 to 20, 27 to 32 is shown as DMSO + E2.

Figures 33 to 48 illustrate the luciferase activity provided by a human mutant ERα or a human normal ERα. The reporter gene, the human mutant ERα gene and the human normal ERα were expressed in the chromosomes of the cell. Figures 33 to 40 illustrate the luciferase activity in the presence of various concentrations of 4-hydroxytamoxifen, ZM189154 or raloxifene as the sole probable agent of stimulating a human mutant ERα or a human normal ERα. Figures 41 to 48 illustrate the luciferase activity in the presence of 100pM of E2 with various concentrations of 4-hydroxytamoxifen, ZM189154 or raloxifene. A stably transformed binary cell was utilized to express in the chromosomes, the reporter gene with the human mutant ERα gene or with the human normal ERα gene. Figures 33 to 40 zero together the luciferase activity provided by the controls, in which the human mutant ERα or the human normal

ERα was in the presence of DMSO (containing no 4-hydroxytamoxifen, raloxifene or ZM189154). Figures 41 to 48 zero together the luciferase activity provided by the controls, in which the human mutant ERα or the human normal ERα was in the presence of a DMSO solution containing 100pM of E2. In zeroing the luciferase activity provided by the human mutant ERα and human normal ERα, the luciferase activity by the controls were set together as 100% luciferase activity. The luciferase activity provided by the controls in Figures 33 to 40 is shown as DMSO. The luciferase activity provided by the controls in Figures 41 to 48 is shown as DMSO + E2.

Figures 49 to 52 illustrate, as a comparative example, the luciferase activity provided by a human mutant ERαK531E or a human normal ERα, in which the reporter gene was transiently expressed in the cell. Figures 49 and 50 illustrate the luciferase activity in the presence of various concentrations of 4-hydrozytamoxifen as the sole probable agent of stimulating a human mutant ERα. Figures 51 and 52 illustrate the luciferase activity in the presence of 100pM of E2 with various concentrations of 4-hydrxytamoxifen. Figures 49 and 50 zero together the luciferase activity of the controls, in which the human normal ERα or the human mutant ERαK531E was in the presence of DMSO (containing no 4-hydrozytamoxifen). Figures 51 and 52 zero together the luciferase activity of the controls, in which the human normal ERα or the human mutant ERαK531E was in the presence of a DMSO solution containing 100pM of E2. In zeroing the luciferase activity provided by the human mutant ERα and human normal ERα, the resulting luciferase activity by the controls is set as 100% luciferase activity. The luciferase activity provided by the controls in Figures 49 and 50 is shown as DMSO. The luciferase activity provided by the controls in Figures 51 and 52 is shown as DMSO + E2.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1. Definitions

AR: means the androgen receptor protein.

E2: means estradiol.

ERα: means an estrogen receptor α protein. Specified mutants of ERα are referred to herein by a letter-number-letter combination following the phrase "mutant ERα", such as by K303R, S309F, G390D, M396V, G415V, G494V, K531E and S578P. In the letter-number-letter combination, the number indicates the relative position of a substituted amino acid in the mutant ERα, the letter preceding the number indicates the amino acid in a normal ERα at the indicated relative position and the letter following the number indicates the substituted amino acid in the provided mutant ERα at the indicated relative position. When there are two substituted amino acids in the mutant ERα, the phrase "mutant ERα" is followed by two letter-number-letter combinations, such as by G390D/S578P.

ERβ: means the estrogen receptor β protein.

GR: means the glucocorticoid receptor protein.

MR: means the mineralocorticoid receptor protein.

PPAR: means the peroxisome proliferator-activated receptor protein.

PR: means the progesterone receptor protein.

PXR: means the pregnane X receptor protein.

TR: means the thyroid hormone receptor protein.

VDR: means the vitamin D receptor protein.

DR1: means the receptor responsive sequence having the following nucleotide sequence:

5'-AGGTCAnAGGTCA-3'

wherein n represents an A, C, T or G.

DR3: means the receptor responsive sequence having the following nucleotide sequence:

5'-AGGTCAnnnAGGTCA-3'

wherein n represents an A, C, T or G.

DR4: means the receptor responsive sequence having the following nucleotide sequence:

5'-AGGTCAnnnnAGGTCA-3'

wherein n represents an A, C, T or G.

ERE: means the estrogen responsive element nucleotide sequence.

MMTV: means the mouse mammary tumor virus

5.2. The cell

The cell of the present invention comprises a chromosome which comprises a reporter gene. The reporter gene in the chromosome comprises an ERE, a TATA sequence and a reporter sequence. In addition, the cell comprises a mutant ERα or a gene encoding the mutant ERα. In this regard, the cell provides a biological system in which the mutant ERα can have an activity for transactivation of the reporter gene. The activity for transactivation of the reporter gene by the mutant ERα in the presence of E2 and a partial anti-estrogen is typically higher than that by a normal ERα in the presence of E2 and the partial anti-estrogen. Alternatively, the activity for transactivation of the reporter gene by the mutant ERα in the presence of the partial anti-estrogen as the sole probable agent of stimulating the mutant ERα is typically higher than that by the normal ERα in the presence of the partial anti-estrogen as the sole probable agent of stimulating the normal ERα.

Typically, the ERE, the TATA sequence and the reporter sequence are organized in the reporter gene to allow the transactivation of the reporter gene. For example, the reporter gene can have the ERE operably upstream from the TATA sequence and the reporter sequence operably downstream from the TATA sequence. If so desired, the reporter gene may additionally contain conventional nucleotide sequences advantageous for the expression of the reporter gene.

The TATA sequence may have the following nucleotide sequence:

5'-TATAA-3'

In a natural cell, the ERE is a receptor responsive sequence that is cognate with a normal ERα. When normal ERα binds to E2 and the normal ERα-E2 complex binds

to the ERE, the normal ERα has an activity for transactivation. In the cell, it is a function of the ERE to bind to the mutant ERα and allow the mutant ERα to have an activity for transactivation of the reporter gene. Typically, such an ERE is encompassed by the following nucleotide sequence:

5'-AGGTCAnnnTGACCTT-3'

wherein n represents an A, G, C or T. Further, a tandem repeat of the ERE in the reporter gene can provide a more efficient activity for transactivation of the reporter gene. A 2 to 5 tandem repeat of the ERE may be used in the reporter gene. As an example of an ERE which can be utilized in the reporter gene, there is mentioned an ERE derived from Xenopus vitellogenin gene (Cell, 57, 1139-1146). The ERE can be prepared for the reporter gene by being chemically synthesized or by being cloned with polymerase chain reaction (PCR) amplification methods.

The reporter sequence in the reporter gene is a reporter sequence naturally foreign to the ERE. As such, the reporter sequence and the ERE are not found together in a natural gene. Further, when such a reporter sequence encodes a reporter protein, the reporter sequence typically encodes a reporter protein that is more or less active in the cell. As examples of the reporter protein, there is mentioned a luciferase, a secretory alkaline phosphatase, a β-galactosidase, a chloramphenicol acetyl transferase, a growth hormone and the like.

Conventional methods may be used to ligate the ERE, the TATA sequence and the reporter sequence. After producing the reporter gene, the reporter gene may be inserted into a chromosome. The reporter gene may be inserted into a chromosome when the reporter gene is introduced into a host cell. Such methods of introducing the reporter gene into a host cell are described below.

The mutant ERα in the cell typically has a particular activity for transactivation of the reporter gene when in the presence of E2 and a partial anti-estrogen or when in the presence of the partial anti-estrogen as the sole probable agent of stimulating the mutant ERα. The activity for transactivation provided by the mutant ERα in the presence of E2 and the partial anti-estrogen is typically higher than that by a normal ERα in the presence of E2 and the partial anti-estrogen. The activity for

transactivation of the reporter gene by the mutant ERα in the presence of the partial anti-estrogen as the sole probable agent of stimulating the mutant ERα is higher than that by the normal ERα in the presence of the partial anti-estrogen as the sole probable agent of stimulating the normal ERα. Since transactivation involves the increase of rate of transcription, such a transactivation by the normal ERα and mutant ERα can be observed by measuring the expression level of the reporter gene. When the expression levels of the reporter gene provided by the mutant ERα and normal ERα are adjusted to be zeroed at identical points, the mutant ERα would provide a higher expression level than that provided by the normal ERα.

Further, it should be noted that the mutant ERα may have the activity for transactivation of the reporter gene inhibited in the presence of the pure anti-estrogen. Such a activity for transactivation for the reporter gene provided by the mutant ERα is similar to the inhibition of the activity for transactivation of the reporter gene provided by the normal ERα in the presence of the pure anti-estrogen.

A normal ERα encompasses the ERα which is reported as most commonly carried in a species, such as human, monkey, mouse, rabbit, rat and the like. For example, a human normal ERα has the amino acid sequence shown in SEQ ID:1. Such a human normal ERα is described in Tora L. et al., EMBO, vol 8 no 7: 1981-1986 (1989).

The partial anti-estrogens typically are not antagonistic to an AF1 region of the normal ERα and are antagonistic to an AF2 region of a normal ERα. The AF2 region of a normal ERα and the AF1 region of a normal ERα are each regions in the normal ERα that are involved in transactivation by the normal ERα (Metzger D. et al., J. Biol. Chem., 270:9535-9542 (1995)).

Such properties of the partial anti-estrogens may be observed, for example, by carrying out the reporter assay described in Berry M. et al., EMBO J., 9:2811-2818 (1990). In such a reporter assay, there is utilized cells in which the AF1 region of an endogenous normal ERα has a strong activity for transactivation, such as chicken embryo fibroblast cells in primary culture (that may be prepared according to the

description, for example, in Solomon, J. J., Tissue Cult. Assoc. Manual., 1:7-11 (1975)). When utilized, the chicken embryo fibroblast are modified so that the modified fibroblasts express therein a gene encoding the normal ERα and so that the modified fibroblasts have the reporter gene (hereinafter referred to as AF1 evaluation fibroblasts). When the AF1 evaluation fibroblasts are exposed with a sufficient amount of a partial anti-estrogen, it can be determined whether the partial anti-estrogen fails to be antagonistic to an AF1 region of a normal ERα. The partial anti-estrogen in such cases increase the expression level of the reporter gene in the AF1 evaluation fibroblasts. Further, the chicken embryo fibroblast cells in primary culture are then modified for a second round so that the second modified fibroblasts express a gene encoding a truncated normal ERα which has the AF1 region deleted and so that the second modified fibroblasts have the reporter gene (hereinafter referred to as AF2 evaluation fibroblasts). When the AF2 evaluation fibroblasts are exposed with a sufficient amount the partial anti-estrogen, it can be determined whether the partial anti-estrogen is antagonistic to an AF2 region of a normal ERα. The partial anti-estrogen in such cases fails to increase the expression level of the reporter gene in the AF2 evaluation fibroblasts.

Examples of such parital anti-estrogens include tamoxifen, 4-hydroxytamoxifen, raloxifene and the like.

The pure anti-estrogen is typically an anti-estrogen which is fully antagonistic to a normal ERα. In this regard, the pure anti-estrogen fails to be partially agonistic to the ERα. In a reporter assay with either the AF1 evaluation fibroblasts or the AF2 evaluation fibroblasts, the pure anti-estrogen provides substantially no activity for transactivation of the reporter gene by the normal ERα or truncated normal ERα therein. As such, the expression level of the reporter gene in such reporter assays with the pure-anti-estrogen and either of the AF1 evaluation fibroblasts or AF2 evaluation fibroblasts does not substantially increase.

Examples of such pure anti-estrogen include ICI 182780 (Wakeling AE et al., Cancer Res., 512:3867-3873 (1991)), ZM 189154 (Dukes M et al., J. Endocrinol., 141:335-341 (1994)) and the like.

The mutant ERα comprises one or more substituted amino acids which confers such an activity for transactivation of the reporter gene in the presence of E2 and the partial anti-estrogen or in the presence of the partial anti-estrogen as the sole probable agent of stimulating the mutant ERα. Typically, the one or more substituted amino acids are present in the mutant ERα at one or more relative positions of from 303 to 578. For example, the mutant ERα may comprise one or more substituted amino acids at one or more relative positions selected from 303, 309, 390, 396, 415, 494, 531, 578 and the like. Typically, such relative positions in the mutant ERα are based on a homology alignment to the amino acid sequence shown in SEQ ID:1.

In general, a homology alignment encompasses an alignment of amino acid sequences based on the homology of the provided amino acid sequences. For example, Table 1 below randomly sets forth a homology alignment with the amino acid sequence shown in SEQ ID:1 (a human normal ERα), a mouse ERα (Genbank Accession No. M38651), a rat ERα(X6) (Genbank Accession No. X61098) and a rat ERα(Y0) (Genbank Accession No. Y00102).

Table 1

```
hERa.txt      1:MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGEVYLDSSKPAVYNYPEGAAY  60
mER.txt       1:MTMTLHTKASGMALLHQIQGNELEPLNRPQLKMPMERALGEVYVDNSKPTVFNYPEGAAY  60
ratER(X6).txt 1:MTMTLHTKASGMALLHQIQGNELEPLNRPQLKMPMERALGEVYVDNSKPAVFNYPEGAAY  60
ratER(Y0).txt 1:MTMTLHTKASGMALLHQIQGNELEPLNRPQLKMPMERALGEVYVDNSKPAVFNYPEGAAY  60

hERa.txt      61:EFNAAAAAMAQ------VYGQTGLPYGPGSEAAAFGSNGLGGFPPLNSVSPSPLMLLHPPP 115
mER.txt       61:EFNAAAAAAAASAP--VYGQSGIAYGPGSEAAAFSANSLGAFPQLNSVSPSPLMLLHPPP 119
ratER(X6).txt 61:EFNAAAAAAAAGASAPVYGQSSITYGPGSEAAAFGANSLGAFPQLNSVSPSPLMLLHPPP 120
ratER(Y0).txt 61:EFNAAAAAAAAGASAPVYGQSSITYGPGSEAAAFGANSLGAFPQLNSVSPSPLMLLHPPP 120

hERa.txt      116:QLSPFLQPHGQQVPYYLENEPSGYTVREAGPPAFYRPNSDNRRQGGRERLASTNDKGSMA 175
mER.txt       120:QLSPFLHPHGQQVPYYLENEPSAYAVRDTGPPAFYRSNSDNRRQNGRERLSSSNEKGNMI 179
ratER(X6).txt 121:HVSPFLHPHGHQVPYYLENEPSAYAVRDTGPPAFYRSNSDNRRQNGRERLSSSSEKGNMI 180
ratER(Y0).txt 121:HVSPFLHPHGHQVPYYLENEPSAYAVRDTGPPAFYRSNSDNRRQNGRERLSSSSEKGNMI 180

hERa.txt      176:MESAKETRYCAVCNDYASGYHYGVMSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRK 235
mER.txt       180:MESAKETRYCAVCNDYASGYHYGVMSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRK 239
ratER(X6).txt 181:MESAKETRYCAVCNDYASGYHYGVMSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRK 240
ratER(Y0).txt 181:MESAKETRYCAVCNDYASGYHYGVMSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRK 240

hERa.txt      236:SCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVGSAGDMRAANLWPSP 295
mER.txt       240:SCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDLEGRVEVGASGDMRAANLWPSP 299
ratER(X6).txt 241:SCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDLEGRVEVGTSGDMRAANLWPSP 300
ratER(Y0).txt 241:SCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDLEGRVEVGTSGDMRAANLWPSP 300
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                                   ✲
hERa.txt         296:LMIKRSKKNSLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELV 355
mER.txt          300:LMIKHTKKNSPALSLTADQMVSALLDAEPPMIYSEYDPSRPFSEASMMGLLTNLADRELV 359
ratER(X6).txt    301:LMIKHTKKNSPALSLTADQMVSALLDAEPPLIYSEYDPSRPFSEASMMGLLTNLADRELV 360
ratER(Y0).txt    301:LMIKHTKKNSPALSLTADQMVSALLDAEPPLIYSEYDPSRPFSEASMMGLLTNLADRELV 360

hERa.txt         356:HMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQG 415
mER.txt          360:HMINWAKRVPGFGDLNLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQG 419
ratER(X6).txt    361:HMINWAKRVPGFGDLNLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQG 420
ratER(Y0).txt    361:HMINWAKRVPGFGDLNLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQG 420

hERa.txt         416:KCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHI 475
mER.txt          420:KCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHI 479
ratER(X6).txt    421:KCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHI 480
ratER(Y0).txt    421:KCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHI 480

hERa.txt         476:HRVLDKITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVP 535
mER.txt          480:HRVLDKITDTLIHLMAKAGLTLQQQHRRLAQLLLILSHIRHMSNKGMEHLYNMKCKNVVP 539
ratER(X6).txt    481:HRVLDKITDTLIHLMAKAGLTLQQQHRRLAQLLLILSHIRHMSNKGMEHLYNMKCKNVVP 540
ratER(Y0).txt    481:HRVLDKINDTLIHLMAKAGLTLQQQHRRLAQLLLILSHIRHMSNKGMEHLYNMKCKNVVP 540
                                                                ✲
hERa.txt         536:LYDLLLEMLDAHRLHAPTSRGGASVEETDQSHLATAGSTSSHSLQKYYITGEAEGFPATV 595
mER.txt          540:LYDLLLEMLDAHRLHAPASRMGVPPEEPSQTQLATTSSTSAHSLQTYYIPPEAEGFPNTI 599
ratER(X6).txt    541:LYDLLLEMLDAHRLHAPASRMGVPPEEPSQSQLTTTSSTSAHSLQTYYIPPEAEGFPNTI 600
ratER(Y0).txt    541:LYDLLLEMLDAHRLHAPASRMGVPPEEPSQSQLTTTSSTSAHSLQTYYIPPEAEGFPNTI 600
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In Table 1, "hERa.TXT" sets forth the amino acid sequence shown in SEQ ID:1, "mER.TXT" sets forth the amino acid sequence of the mouse ERα, "ratER(X6).TXT" sets forth the amino acid sequence of the rat ERα(X6) and "ratER(Y0)" sets forth the amino acid sequence of rat ERα(Y0), wherein amino acids sequences thereof are set forth using one letter abbreviations of the amino acids. This alignment was prepared using a commercially available software GENETYX-WIN SV/R ver. 4.0 (Software Development Co.). The symbol "*" indicates the amino acids located at relative positions 303 and 578.

The relative positions under the homology alignment correspond to the absolute positions of the amino acid sequence shown in SEQ ID:1. For example, relative position 303 encompasses under the homology alignment, the amino acid in the mutant ERα aligned with the 303rd amino acid from the N-terminus in the amino acid sequence shown in SEQ ID:1. Further, a relative position 578 encompasses under the homology alignment, the amino acid in the mutant ERα aligned with the 578th amino acid from the N-terminus in the amino acid sequence shown in SEQ ID:1. In reference to Table 1, examples of relative position 303 include the lysine that is the 303rd amino acid from the amino terminus in the amino acid sequence shown in SEQ ID:1, the lysine that is the 307th amino acid from the amino terminus in the amino acid sequence of the mouse ERα, the lysine that is the 308th amino acid from the amino terminus in the amino acid sequence of rat ERα(X6) and the lysine that is the 308th amino acid from the amino terminus in the amino acid sequence of the rat ERα(Y0). Further, examples of the relative position 578 in reference to Table 1 include the serine that is the 578th amino acid from the amino terminus in the amino acid sequence shown in SEQ ID NO: 1, the serine that is the 582th amino acid from the amino terminus in the amino acid sequence of the mouse ERα, the serine that is the 583th amino acid from the amino terminus in the amino acid sequence of the rat ERα(X6) and the serine that is the 583th amino acid from the amino terminus in the amino acid sequence of rat ERα (Y0).

In this regard, the homology alignment in connection with the present invention aligns the amino acid sequence shown in SEQ ID:1 with an amino acid sequence encoding mutant ERα, based on the homology of the mutant ERα and the amino acid sequence shown SEQ ID:1. When aligning the amino acid sequence of a mutant ERα in the homology alignment to amino acid sequence SEQ ID:1, such a mutant ERα typically has at least an 80% homology with the amino acid sequence shown in SEQ ID:1.

The mutant ERα can be derived from an animal such as a mammal. Examples of such mammals include human, monkey, rabbit, rat, mouse and the like. For the human mutant ERα, the mutant ERα generally has an amino acid length of 595 amino acids.

In having the substituted amino acid at relative position 303, the mutant ERα may be derived from changing the lysine present at relative position 303 in a normal ERα into a substituted amino acid. In such cases, the mutant ERα may have the substituted amino acid at relative position 303 be arginine, such as a mutant ERα K303R. The human mutant ERα K303R has the amino acid sequence shown in SEQ ID:2.

In having the substituted amino acid at relative position 309, the mutant ERα may be derived from changing the serine present at relative position 309 in a normal ERα into a substituted amino acid. In such cases, the mutant ERα may have the substituted amino acid at relative position 309 be phenylalanine, such as a mutant ERα S309F. The human mutant ERα S309F has the amino acid sequence shown in SEQ ID:3.

In having the substituted amino acid at relative position 390, the mutant ERα may be derived from changing the glycine present at relative position 390 in a normal ERα into a substituted amino acid. In such cases, the mutant ERα may have the substituted amino acid at relative position 390 be aspartic acid, such as a mutant ERα G390D. The human mutant ERα G390D has the amino acid sequence shown in SEQ ID:4.

In having the substituted amino acid at relative position 396, the mutant ERα may be derived from changing the methionine present at relative position 396 in a normal ERα into a substituted amino acid. In such cases, the mutant ERα may have the substituted amino acid at relative position 396 be valine, such as a mutant ERα M396V. The human mutant ERα M396V has the amino acid sequence shown in SEQ ID:5.

In having the substituted amino acid at relative position 415, the mutant ERα may be derived from changing the glycine present at relative position 415 in a normal ERα into a substituted amino acid. In such cases, the mutant ERα may have the substituted amino acid at relative position 415 be valine, such as a mutant ERα G415V. The human mutant ERα G415V has the amino acid sequence shown in SEQ ID:6.

In having the substituted amino acid at relative position 494, the mutant ERα

may be derived from changing the glycine present at relative position 494 in a normal ERα into a substituted amino acid. In such cases, the mutant ERα may have the substituted amino acid at relative position 494 be valine, such as a mutant ERα G494V. The human mutant ERα G494V has the amino acid sequence shown in SEQ ID:7.

In having the substituted amino acid at relative position 531, the mutant ERα may be derived from changing the lysine present at relative position 531 in a normal ERα into a substituted amino acid. In such cases, the mutant ERα may have the substituted amino acid at relative position 531 be glutamic acid, such as a mutant ERα K531E. The human mutant ERα K531E has the amino acid sequence shown in SEQ ID:8.

In having the substituted amino acid at relative position 578, the mutant ERα may be derived from changing the serine present at relative position 578 in a normal ERα into a substituted amino acid. In such cases, the mutant ERα may have the substituted amino acid at relative position 578 be proline, such as mutant ERα S578P. The human mutant ERα S578P has the amino acid sequence shown in SEQ ID:9.

In having the substituted amino acid at relative position 390 and 578, the mutant ERα may be derived from changing the glycine present at relative position 390 in a normal ERα into a substituted amino acid as well as changing the serine present at relative position 578 in the normal ERα into another substituted amino acid. In such cases, the mutant ERα may have the substituted amino acid at relative position 390 be aspartic acid and the substituted amino acid at relative position 578 be proline, such as mutant ERα G390D/S578P. The human mutant ERα G390D/S578P has the amino acid sequence shown in SEQ ID:10.

To provide the mutant ERα, the cell may express a gene encoding the mutant ERα, according to the standard genetic code which is well known. Such a mutant ERα gene typically comprises a polynucleotide which encodes the mutant ERα and a promoter. The mutant ERα gene can be isolated from tissue sample. Further, the mutant ERα gene may be produced by using mutagenesis techniques to mutagenize a polynucleotide encoding a normal ERα to encode the mutant ERα and by operably

linking a promoter upstream from the resulting polynucleotide encoding the mutant ERα. The mutagenesis techniques, such as site-directed mutagenesis, may be utilized to introduce the one or more mutations into the normal ERα polynucleotide and provide a mutant ERα polynucleotide. The human normal ERα polynucleotide having the nucleotide sequence described in Tora L. et al. EMBO J., vol 8 no 7:1981-1986 (1989) is utilized in the case of mutagenizing the human normal ERα polynucleotide.

The promoter in the mutant ERα gene initiates transcription so that the mutant ERα can be expressed to provide the mutant ERα in the cell. In this regard, a promoter capable of functioning in the cell is usually operably linked upstream to a polynucleotide encoding the mutant ERα. For instance, where the cell is derived from an animal host cell or fission yeast host cell, examples of the promoter may include Rous sarcoma virus (RSV) promoter, cytomegalovirus (CMV) promoter, early and late promoters of simian virus (SV40), MMTV promoter and the like. Where the cells are derived from budding yeast host cell, examples of the promoter may include ADH1 promoter and the like.

In using the mutagenesis techniques, a polynucleotide encoding normal ERα can be isolated and then the isolated normal ERα polynucleotide can be mutagenized by using oligonucleotides. The resulting mutant ERα polynucleotide can then be utilized to produce the mutant ERα gene.

Oligonucleotides are designed and synthesized to specifically amplify a cDNA encoding a normal ERα from a cDNA library or the cDNAs of an animal. Such oligonucleotides can be designed, based on a well known nucleotide sequence encoding the normal ERα, such as the normal ERα nucleotide sequences found in documents, such as Tora L. et al. EMBO J., vol 8 no 7:1981-1986 (1989), or in databases such as in Genbank. As such normal ERα nucleotide sequences, there can be utilized a normal ERα nucleotide sequence derived from human, monkey, rabbit, rat, mouse or the like. The designed oligonucleotides can then be synthesized with a DNA synthesizer (Model 394, Applied Biosystems). A polymerase chain reaction (PCR) amplification may then be utilized to isolate the normal ERα polynucleotide from the cDNA library or cDNAs.

For human normal ERα gene, the oligonucleotides depicted in SEQ ID:11 and SEQ ID:12 may be utilized to PCR amplify the human normal ERα polynucleotide having the nucleotide sequence described in Tora L. et al. EMBO J., vol 8 no 7:1981-1986 (1989).

The cDNAs can be derived from animal tissue (such as human, monkey, rabbit, rat, or mouse) according to genetic engineering techniques described in J. Sambrook, E. F. Frisch, T. Maniatis, "Molecular Cloning, 2nd edition", Cold Spring Harbor Laboratory, 1989. In such techniques, the RNAs in an animal tissue, such as liver or uterus, are collectively extracted therefrom and the RNAs are collectively reverse transcribed into the cDNAs of the animal. For example, the animal tissue is first homogenized in a buffer containing a protein denaturing agent such as guanidine hydrochloride or guanidine thiocyanate. Reagents such as a mixture containing phenol and chloroform (hereinafter referred to as phenol-chloroform) are further added to denature proteins resulting from homogenizing the animal tissue. After removing the denatured proteins by centrifugation, the RNAs are collectively extracted from the recovered supernatant fraction. The RNAs can be collectively extracted by methods such as the guanidine hydrochloride/phenol method, SDS-phenol method, the guanidine thiocyanate/CsCl method and the like. ISOGEN (Nippon Gene) is an example of a commercially available kit which is based on such methods of collectively extracting the RNAs. After collectively extracting the RNAs, oligo-dT primers are allowed to anneal to the poly A sequence in the RNAs to collectively reverse transcribe the RNAs as a template. A reverse transcriptase can be utilized to collectively reverse transcribe the RNAs into single-stranded cDNAs. The cDNAs can be synthesized from the single-strand cDNAs by using E. coli DNA polymerase I with the above single-stranded cDNAs. In using E. coli DNA polymerase I, E. coli RNase H is also used to produce primers, which allow E. coli DNA polymerase I to operate more efficiently. The cDNAs can be purified by using conventional purifying procedures, for example, by phenol-chloroform extraction and ethanol precipitation. Examples of commercially available kits based on such methods include cDNA Synthesis System Plus (Amersham Pharmacia Biotech), TimeSaver cDNA Synthesis kit (Amerham Pharmacia Biotech)

and the like.

The normal ERα polynucleotide is then isolated from the cDNAs. Isolation procedures which may be utilized to isolate the normal ERα polynucleotide may include using PCR amplification. The PCR amplification typically amplifies the normal ERα polynucleotide from the cDNAs. The PCR mixture in the PCR amplification may contain a sufficient amount of the cDNAs, a sufficient amount of the forward and reverse oligonucleotides, a heat tolerant DNA polymerase (such as LT-Taq polymerase (Takara Shuzo)), dNTPs (dATP, dTTP, dGTP, dCTP) and a PCR amplification buffer. In a PCR mixture amplifying a human normal ERα polynucleotide, there may be utilized 10 ng of the cDNAs and 10 pmol of the each of the forward and reverse oligonucleotides (SEQ ID:11 and SEQ ID:12). The PCR mixture in the PCR amplification then undergoes an incubation cycle for annealing, elongation and denaturing. For example, the PCR amplification may have repeated 35 times with a thermal cycler such as PCR System 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute and then an incubation at 68℃ for 3 minutes. After the PCR amplification with the cDNAs, the whole amount of resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After confirming the presence of a band therein comprising the normal ERα polynucleotide, the normal ERα polynucleotide is recovered from the low melting point agarose gel.

As the cDNA libraries, there can be utilized a commercially available cDNA library derived from an animal, such as QUICKClone cDNAs (manufactured by Clontech). The cDNA library may then be isolated as described above.

The nucleotide sequence of the recovered normal ERα polynucleotide can be confirmed by preparing a sample of the normal ERα polynucleotide for direct sequencing. Also, DNA fluorescence sequencing techniques may be utilized to sequence the normal ERα polynucleotide. In this regard, to prepare the sample of the normal ERα polynucleotide, there can be utilized commercially available reagents for fluorescence sequencing such as Dye Terminator Sequencing kit FS (Applied Biosystems). The fluorescence sequencing of the normal ERα polynucleotide may be

conducted with an autosequencer such as ABI autosequencer (Model 377, Applied Biosystems). Further , the normal ERα polynucleotide may be manually sequenced (Biotechniques, 7, 494(1989)).

For convenience, the isolated normal ERα polynucleotide can be inserted into a vector capable of replicating in a host such as E. coli. For example, about 1 μg of isolated normal ERα polynucleotide may have the ends thereof blunted by a treatment with DNA blunting kit (Takara Shuzo), when the provided isolated normal ERα polynucleotide has uneven ends. A T4 polynucleotide kinase may then be used to phosphorylate the ends of the blunt-ended normal ERα polynucleotide. After phenol treatment, the normal ERα polynucleotide is purified by ethanol precipitation and may be inserted into a vector capable of replication in E. coli. The E. coli vector comprising the normal ERα polynucleotide may be cloned into E. coli host cells.

The E. coli vector comprising the normal ERα polynucleotide may then be isolated from the cloned E. coli cells. The isolated E. coli vector comprising the normal ERα polynucleotide is then used as a template to mutagenize, i.e., introduce nucleotide substitutions, into the normal ERα polynucleotide, such that the resulting mutant ERα polynucleotide contains a variant codon encoding the substituted amino acid at the desired relative position.

The desired nucleotide substitutions may be introduced into the normal ERα polynucleotide according to the site-directed mutagenesis methods described in J. Sambrook, E. F., Frisch, T. Maniatis, "Molecular Cloning 2nd edition", Cold Spring Harbor Laboratory, 1989, or the site directed mutagenesis methods described in McClary JA et al., Biotechniques 1989(3): 282-289. For example, the desired nucleotide substitutions may be introduced into the normal ERα polynucleotide by using a commercially available kit, such as QuickChange Site-Directed Mutagenesis kit manufactured by Stratagene. Typically, such site-directed mutagenesis methods utilize oligonucleotides which introduce the desired nucleotide substitutions therein. In relation to the QuickChange Site-Directed Mutagenesis kit, the following describes in more detail the site-directed mutagenesis methods utilized with the normal ERα polynucleotide.

The QuickChange Site-Directed Mutagenesis kit utilizes two oligonucleotides to achieve the desired nucleotide substitution into the normal ERα polynucleotide. As such a combination of the two oligonucleotides, there may be utilized for human normal ERα polynucleotide, the combination of oligonucleotides selected from the combination including the oligonucleotide depicted in SEQ ID:13 with the oligonucleotide depicted in SEQ ID:14, the combination including the oligonucleotide depicted in SEQ ID:15 with the oligonucleotide depicted in SEQ ID:16, the combination including the oligonucleotide depicted in SEQ ID:17 with the oligonucleotide depicted in SEQ ID:18, the combination including the oligonucleotide depicted in SEQ ID:19 with the oligonucleotide depicted in SEQ ID:20, the combination including the oligonucleotide depicted in SEQ ID:21 with the oligonucleotide depicted in SEQ ID:22, the combination including the oligonucleotide depicted in SEQ ID:23 with the oligonucleotide depicted in SEQ ID:24, the combination including the oligonucleotide depicted in SEQ ID:25 with the oligonucleotide depicted in SEQ ID:26 or the combination including the oligonucleotide depicted in SEQ ID:27 with the oligonucleotide depicted in SEQ ID:28. Table 2 below shows the relative position of the amino acid encoded at the locus of the nucleotide substitution and the resulting variant codons from utilizing such combinations of the oligonucleotides.

Table 2

SEQ ID of oligonucleotides	relative position	nucleotide sequence in encoding normal ERα	variant codon in encoding mutant ERα
13 & 14	303	AAG (lysine)	AGG (arginine)
15 & 16	309	TCC (serine)	TTC (phenylalanine)
17 & 18	390	GGT (glycine)	GAT (aspartic acid)
19 & 20	396	ATG (methionine)	GTG (valine)
21 & 22	415	GGA (glycine)	GTA (valine)
23 & 24	494	GGC (glycine)	GTC (valine)
25 & 26	531	AAG (lysine)	GAG (glutamic acid)
27 & 28	578	TCC (serine)	CCC (proline)

The achieved mutant ERα polynucleotide can be sequenced to confirm that the desired nucleotide substitution has been introduced into the normal ERα polynucleotide.

To produce the cell, the mutant ERα gene and the reporter gene are usually introduced into a host cell. The reporter gene is introduced into the host cell so that the reporter gene is inserted into a chromosome of the host cell. The mutant ERα gene is introduced into the host cell for transient expression or is inserted into a chromosome of the host cell. When inserting the mutant ERα gene into a chromosome of the host cell, the mutant ERα gene and reporter gene may be introduced into one chromosome or the mutant ERα gene may be inserted into chromosome other than the chromosome utilized for the reporter gene.

The host cell typically fails have an expressed normal or mutant ERα. Examples of the host cells may include budding yeast cells such as CG1945 (Clontech), animal cells such as HeLa cells, CV-1 cells, Hepa1 cells, NIH3T3 cells, HepG2 cells, COS1 cells, BF-2 cells, CHH-1 cells and insect cells and the like.

The mutant ERα gene and the reporter gene may be inserted into vectors, so that the mutant ERα gene and the reporter gene can be introduced into the host cell. Such vectors typically have a replication origin so that the vector can be replicated in the cell. If so desired, the vector may also have a selective marker gene.

Where the budding yeast cell is used as a host cell, examples of the vector may include plasmid pGBT9, pGAD424, pACT2 (Clontech) and the like. Where mammalian cells are used as host cells, examples of the vector may include plasmids

such as pRc/RSV, pRc/CMV (Invitrogen), vectors containing an autonomous replication origin derived from viruses such as bovine papilloma virus plasmid pBPV (Amersham Pharmacia Biotech), EB virus plasmid pCEP4 (Invitrogen) and the like.

When producing a vector encoding the mutant ERα (hereinafter referred to as the mutant ERα vector), it is preferable for the vector to additionally contain the promoter so that the mutant ERα polynucleotide can be inserted into the vector to produce together the mutant ERα gene with the mutant ERα vector. Likewise, when producing a vector encoding the reporter gene (hereinafter referred to as the reporter vector), it is preferable for the vector to contain a TATA sequence or an ERE so that the reporter gene can be produced together with the reporter vector.

When producing the mutant ERα vector together with the mutant ERα gene for an animal host cell, pRc/RSV or pRc/CMV can be utilized. The plasmids pRc/RSV and pRc/CMV contain a promoter which can function in the cell, when derived from an animal host cell, and a cloning cite operably downstream from the promoter. In this regard, the mutant ERα vector can be produced together with the mutant ERα gene by inserting the mutant ERα polynucleotide into pRc/RSV or pRc/CMV at the cloning site. Since pRc/RSV and pRc/CMV also contain an autonomous replication origin of SV40 (ori), pRc/RSV and pRc/CMV may be used to introduce the mutant ERα gene into the animal host cells transformed with ori(-) SV40 genome, if so desired. As such animal host cells transformed with ori(-) SV40 genome, there is mentioned COS cells. When introduced into such animal host cells transformed with ori(-) SV40 genome, the mutant ERα vector produced from pRc/RSV or pRc/CMV can increase to a fairly large copy number therein such that the mutant ERα gene can be expressed in a large amount.

When introducing the mutant ERα vector into a budding yeast host cell, it is preferable to utilize pACT2 to produce the mutant ERα vector. Since pACT2 carries an ADH1 promoter, the mutant ERα gene can be produced together with the mutant ERα vector by inserting the mutant ERα polynucleotide downstream of the ADH1 promoter. In such cases, a the mutant ERα vector can express the mutant ERα gene in a large

amount.

Conventional techniques can be used for introducing the mutant ERα gene, according to the type of host cell. For example, the calcium phosphate method, DEAE-dextran method, electroporation, lipofection or the like may be use where mammalian or insect cells are used as host cells. Where yeast cells are used as host cells, there may be used a lithium method such as a method using the Yeast transformation kit (Clontech) or the like.

Furthermore, where the mutant ERα gene is introduced into the host cell as viral DNA, the mutant ERα gene may be introduced into host cells not only by the techniques as described above, but also by infecting the host cells with recombinant virions containing the viral forms of the reporter gene and the mutant ERα gene. For example, viruses such as vaccinia virus may utilized for animal host cells and where insect animal cells are used as host cells, there may be utilized insect viruses such as baculovirus.

When the mutant ERα vector or the reporter vector comprises the selective marker gene, as described above, the selective marker gene may be employed to clone the cell of the present invention. In such cases, the selective marker gene can be utilized to confer a drug resistance to a selective drug exhibiting lethal activity on the cell. The cell in this regard may then be cloned by culturing the cell in a medium supplemented with said selective drug. Exemplary combinations of the selective marker gene and selective drug include a combination of neomycin resistance-conferring selective marker gene and neomycin, a combination of hygromycin resistance-conferring selective marker gene and hygromycin, a combination of blasticidin S resistance-conferring selective marker gene and blasticidin S and the like. In a case wherein the selection marker gene encodes a nutrient which complements the auxotrophic properties of the cell, the cell may be cultured using a minimal medium that substantially contains none of the nutrient. Furthermore, an assay measuring an estrogen binding activity may also used to clone the cell.

In introducing the reporter gene into the host cell, the reporter gene is usually introduced in a linearized form. The linearized reporter gene may allow the reporter

gene to be inserted into the chromosome of the host cell. When utilizing the reporter vector, the reporter vector can be linearized by a restriction digestion. The lipofection method may be utilized to introduce the linearized reporter gene into the host cell.

Further, it should be noted that the reporter gene may be introduced into the host cell, before introducing the mutant ERα gene to provide a stably transformed cassette cell. The stably transformed cassette cell stably comprises the reporter gene in a chromosome thereof such that the reporter gene can be genetically handed down to progeny generations. To produce the stably transformed cassette cell, the reporter gene may be introduced into the chromosome of a host cell and the host cell may be cultured for several weeks. After culturing for several weeks, the stably transformed cassette cell can be cloned by employing the selective marker gene, when utilized. For example, the transformed host cells may be continuously cultured for several weeks in a medium supplemented with the selective drug to clone the stable transformed cassette cell. The mutant ERα gene may then be introduced into the stably transformed cassette cell to produce the cell.

Furthermore, the mutant ERα gene may also be introduced into the host cell with the reporter gene so that the host cell is stably transformed with the reporter gene and the mutant ERα gene.

The cell can be utilized to screen for a compound useful for treating a disorder of the mutant ERα. Such a disorder of a mutant ERα may be a disorder which involves an aberrant transactivation by the mutant ERα, such as breast cancer. To screen such a compound, the cell is exposed with an efficient amount of a test compound suspected of being antagonistic or agonistic to the mutant ERα and the transactivation level of the reporter gene is measured.

The cell is typically exposed with a sufficient amount of the test compound for one to several days. The cell can be exposed with the test compound under agonistic conditions or antagonistic conditions directed to the mutant ERα. The agonistic conditions typically have the assay cell exposed to the test compound as the sole agent probable of stimulating the mutant ERα. The antagonistic conditions typically have the assay cell exposed to the test compound and E2.

After exposure, the transactivation level of the reporter gene is measured by measuring the expression level of the reporter gene. In such cases, the reporter protein or the reporter RNA (encoded by the reporter sequence) is stored in the cell or is secreted from the cell so that the expression level can be measured therewith. The expression level of the reporter gene can be measured by a Northern blot analysis, by a Western blot analysis or by measuring the activity level of the reporter protein. The activity level of the reporter protein typically indicates the level at which the reporter gene is expressed.

For example, when the reporter gene encodes luciferase as the reporter protein, the expression level of the reporter gene can be measured by the luminescence provided by reacting luciferin and luciferase. In such cases, a crude cell extract is produced from the cells and luciferin is added to the crude cell extract. The luciferin may be allowed to react with the luciferase in the cell extract at room temperature. The luminescence from adding luciferin is usually measured as an indicator of the expression level of the reporter gene, since the crude cell extract produces a luminescence at a strength proportional to the level of luciferase expressed in the cell and present in the crude cell extract. A luminometer may be utilized to measure the luminescence in the resulting crude cell extract.

The measured transactivation level can then be compared with a control to evaluate the agonistic or antagonistic effect of the test compound. Such a control in screening the test compound can be the expected transactivation level of the reporter gene when the cell is not exposed to the test compound. When the transactivation level of the reporter gene by the mutant ERα is higher than the control under the agonistic conditions, the test compound is evaluated as an agonist directed to the mutant ERα.

Alternatively, when the cell is exposed to E2 and the test compound under the antagonistic conditions, the test compound can be evaluated as an antagonist directed to the mutant ERα. In such cases, the control can be the expected transactivation level of the reporter gene by the mutant ERα in the presence of an equivalent amount of E2. When the transactivation level of the reporter gene by the mutant ERα is lower than the control, the test compound is evaluated as being antagonistic to the mutant ERα.

Such a test compound agonistic or antagonistic to the mutant ERα can then be selected as a compound useful for treating a disorder of the mutant ERα. In such cases, the test compound which provides an transactivation level of the reporter gene which is significantly higher than the control is usually selected when the cell is exposed under the agonistic conditions. The test compound which provides an transactivation level of the reporter gene which is significantly lower than the control is usually selected when the cell is exposed under the antagonistic conditions.

Furthermore, compounds for treating disorders of normal ligand dependent transcription factors can be screened. In such cases, a gene encoding the normal ligand dependent transcription factor, instead of the mutant ERα gene, is introduced into the host cell. Examples of such normal ligand dependent transcription factors include a normal ERβ (Genbank Accession No. AB006590), a normal AR (Genbank Accession No. M23263), a normal GR (Genbank Accession No. M10901), a normal TRα (M24748), a normal PR (Genbank Accession No. 15716), a normal PXR (Genbank Accession No. AF061056), a normal lipophilic vitamin receptor such as a normal VDR (Genbank Accession No. J03258), a normal RAR (Genbank Accession No. 06538), a normal MR (Genbank Accession No. M16801), a normal PPAR γ (Genbank Accession No. U79012) and the like. The reporter gene in such cases comprises an appropriate receptor responsive sequence cognate with the normal ligand dependent transcriptional factor, instead of the ERE.

5.3. The diagnosis methods

The diagnosis methods of the present invention involve diagnosing the phenotype of a test ERα or the genotype of a polynucleotide encoding the test ERα. In the genotype diagnosis methods, it can be determined whether the polynucleotide encoding the test ERα contains a variant codon therein which provides for the one or more substituted amino acids which confer the activity for transactivation of the reporter gene, as described in the above 5.2. In the phenotype diagnosis methods, it can be determined whether the test ERα contains one or more substituted amino acids therein which confer the activity for transactivation of the reporter gene as described in

the above 5.2.

The genotype diagnosis methods typically involve preparing the test ERα polynucleotide, searching for the variant codon and determining the mutation in the variant codon, if present. Examples of such genotype diagnosis methods include PCR amplification and nucleotide sequencing methods, single strand conformation polymorphism (SSCP) methods, restriction fragment length polymorphism (RFLP) methods, hybridization methods and the like.

The test ERα polynucleotide can be prepared for the genotype diagnosis methods by preparing test genomic DNAs or test cDNA. In such cases, test genomic DNAs or test cDNAs, which contain the test ERα polynucleotide, are collectively prepared from a test sample obtained from a test animal, such as a test human. Such a test sample may be obtained from non-surgical methods, from surgical methods such as from a fine needle or from a biopsy or the like. Examples of such test samples include the cellular tissue of the test mammal, such as hair, peripheral blood, oral epithelial tissue, liver, prostate, ovaries, uterus, mammary gland or the like, from which test genomic DNAs or test cDNAs can be extracted.

For example, the test genomic DNAs can be prepared according to the methods described in TAKARA PCR Technical news No. 2 (Takara Shuzo, 1991.9). In such cases, a test sample of 2 to 3 hairs from a test mammal are washed with sterile water and ethanol and are cut into 2 to 3 mm in length. The test cells in the hairs are then lysed with a sufficient amount, such as 200 μl, of BCL buffer (10 mM of Tris-HCl (pH 7.5), 5 mM of $MgCl_2$, 0.32 M of sucrose, 1% of Triton X-100). The test genomic DNAs therefrom are washed from unnecessary proteins by adding and mixing Proteinase K and SDS to the lysed test cells to amount to final concentrations of 100 μl/ml and 0.5% (w/v), respectively. After incubating the reaction mixture at 70°C, the test genomic DNAs can be purified by a phenol-chloroform extraction.

Additionally, when the test sample is peripheral blood, test genomic DNAs can be collectively obtained, for example, by processing the test sample with DNA-Extraction kit (Stratagene).

Also, when the test sample is obtained from a biopsy, the test cDNAs may be

prepared from the test sample by collectively reverse transcribing the RNAs in the cellular tissue. The RNAs can be collectively obtained from the cellular tissue by using TRIZOL reagent (Gibco), and preferably when the cellular tissue is still fresh.

Furthermore, the test genomic DNAs can be prepared according to the methods described in M. Muramatsu "Labo-Manual-Idenshi-Kogaku" (Maruzen, 1988).

Even furthermore, the test cDNAs may be prepared according to the genetic engineering techniques described in J. Sambrook, E.F. Frisch, T. Maniatis, "Molecular Cloning 2nd edition", Cold Spring Harbor Laboratory, 1989, as described in the above 5.2.

When searching for the variant codon in the genotype diagnosis methods, a searching region in the test ERα polynucleotide typically includes codons therein which are suspected to be the variant codon. As such, the searching region in the test ERα polynucleotide may include the codons in the test ERα polynucleotide which encode the amino acids in the test ERα at relative positions 303 to 578. For example, such genotype diagnosis methods may have the searching region include a codon in the test ERα polynucleotide which encode an amino acid at relative positions selected from 303, 309, 390, 396, 415, 494, 531, 578 and the like.

The PCR amplification and sequencing methods as well as the SSCP methods may then use the prepared test cDNAs or the test genomic DNAs to specifically PCR amplify the searching regions in the test ERα polynucleotide therefrom. Search oligonucleotides can be utilized to specifically PCR amplify from the test cDNAs or test genomic DNAs, the searching regions present in the test ERα polynucleotide.

The search oligonucleotides in this PCR amplification are typically designed to specifically PCR amplify the searching region in the test ERα polynucleotide. The search oligonucleotides may have a size of from 8 to 50bp, preferably 15 to 40 bp, and may have a GC content of 30% to 70%. Such search oligonucleotides may be synthesized with a DNA synthesizer using the β-cyanoethyl phosphoamidide methods, thiophosphite methods or the like. Further, the search oligonucleotides may be unlabeled, non-radioactively labeled, radiolabeled such as with ^{32}P or the like. The PCR amplification typically utilizes a combination of a forward search oligonucleotide and a

reverse search oligonucleotide to specifically PCR amplify the searching region in the test ERα polynucleotide. Examples of such combinations of forward and reverse search oligonucleotides for a human test ERα polynucleotide are shown below in Table 3, in connection with the relative position of the amino acid encoded in the searching region.

Table 3

SEQ IDs depicting the search oligonucleotides		relative position
Forward search oligonucleotide	Reverse search oligonucleotide	
SEQ ID:29, SEQ ID:30, SEQ ID:31, SEQ ID:32 or SEQ ID:33	SEQ ID:34, SEQ ID:35, SEQ ID:36, SEQ ID:37 or SEQ ID:38	303
SEQ ID:39, SEQ ID: 40, SEQ ID:41, SEQ ID:42 or SEQ ID:43	SEQ ID:44, SEQ ID: 45, SEQ ID:46, SEQ ID:47 or SEQ ID:48	309
SEQ ID:49, SEQ ID: 50, SEQ ID:51, SEQ ID:52 or SEQ ID:53	SEQ ID:54, SEQ ID: 55, SEQ ID:56, SEQ ID:57 or SEQ ID:58	390
SEQ ID:59, SEQ ID: 60, SEQ ID:61, SEQ ID:62 or SEQ ID:63	SEQ ID:64, SEQ ID: 65, SEQ ID:66, SEQ ID:67 or SEQ ID:68	396
SEQ ID:69, SEQ ID: 70, SEQ ID:71, SEQ ID:72 or SEQ ID:73	SEQ ID:74, SEQ ID: 75, SEQ ID:76, SEQ ID:77 or SEQ ID:78	415
SEQ ID:79, SEQ ID: 80, SEQ ID:81, SEQ ID:82 or SEQ ID:83	SEQ ID:84, SEQ ID: 85, SEQ ID:86, SEQ ID:87 or SEQ ID:88	494
SEQ ID:89, SEQ ID: 90, SEQ ID:91, SEQ ID:92 or SEQ ID:93	SEQ ID:94, SEQ ID: 95, SEQ ID:96, SEQ ID:97 or SEQ ID:98	531
SEQ ID:99, SEQ ID: 100, SEQ ID:101, SEQ ID:102 or SEQ ID:103	SEQ ID:104, SEQ ID: 105, SEQ ID:106, SEQ ID:107 or SEQ ID:108	578

The searching regions in the test ERα polynucleotide can be specifically PCR amplified from the test cDNAs or the test genomic DNAs according to the methods described in Saiki et al., Science, vol. 230, pp. 1350-1354 (1985). The PCR mixture in this PCR amplification may contain 1.5 mM to 3.0 mM magnesium chloride, heat tolerant DNA polymerase, dNTPs (dATP, dTTP, dGTP, and dCTP), one of the forward search oligonucleotides in combination with one of the reverse search oligonucleotides and the test genomic DNAs or test cDNAs. In this PCR amplification, there may be repeated 20 to 50 times, preferably 25 to 40 times, an incubation cycle entailing a denaturation incubation, an annealing incubation and an elongation incubation. The denaturation incubation may incubate the PCR mixture at 90°C to 95°C, and preferably

at 94°C to 95°C, for 1 min to 5 min, and preferably for 1 min to 2 min. The annealing incubation following the denaturing incubation may incubate the PCR mixture at 30°C to 70°C, and preferably at 40°C to 60°C, for 3 seconds to 3 minutes, and preferably for 5 seconds to 2 minutes. The elongation incubation following the denaturing incubation may incubate the PCR mixture at 70°C to 75°C, and preferably at 72°C to 73°C, for about 15 seconds to 5 minutes, and preferably for 30 seconds to 4 minutes.

When utilizing the PCR amplification and nucleotide sequencing methods, the genotype diagnosis methods may then entail subjecting the resulting PCR mixture to low melting point agarose gel electrophoresis. The amplified polynucleotide encoding the searching region (hereinafter referred to as searching region polynucleotide) is recovered from the low melting point agarose gel and is sequenced to provide a nucleotide sequence of the searching region polynucleotide.

The mutation in the variant codon, if present, may then be determined by sequencing the searching region polynucleotide and by determining the mutation in the nucleotide sequence. In sequencing the searching region polynucleotide, there may be utilized the direct sequencing methods or an automated sequencing method. Examples of the direct sequencing methods include manual sequencing methods (Maxam Gilbert method described in Maxam, A.M. & W. Gilbert, Proc. Natl. Acad. Sci. USA, 74, 560, 1977), the Sanger method (described in Sanger, F. & A. R. Coulson, J. Mol. Biol., 94, 441, 1975 as well as Sanger, F., Nicklen, and A. R., Coulson, Proc. Natl. Acad. Sci. USA., 74, 5463, 1977), the methods described in BioTechniques, 7, 494 (1989) and the like. When an automated DNA sequencer such as ABI autosequencer (Model 377,Applied Biosystems) is used, an appropriate DNA sequencing kit such as ABI Big Dye terminator cycle sequencing ready reaction kit can be used to prepared the searching region for the automated DNA sequencer. After sequencing, the nucleotide sequence of the searching region polynucleotide may then be compared to a nucleotide sequence encoding a normal ERα to determine the mutation in the variant codon, if present, in the searching region.

When utilizing the SSCP methods, the resulting PCR mixture is subjected to a native polyacrylamide gel electrophoresis according to the methods described in Hum.

Mutation, vol. 2, p. 338. In such cases, it is preferable that the PCR amplification above utilize the radiolabeled oligonucleotides so that the searching region polynucleotide is radiolabeled and the searching region polynucleotide can be detected in the native polyacrylamide gel by employing the radioactivity thereof. In such SSCP methods, the radiolabeled searching region polynucleotide can be heat-denatured into single strand polynucleotides and subjected to the native polyacrylamide gel electrophoresis in a buffer to separate each of the single strand polynucleotides. Examples of buffers which may be utilized in the native polyacrylamide gel electrophoresis include Tris-phosphate (pH 7.5-8.0), Tris-acetate (pH 7.5-8.0), Tris-borate (pH 7.5-8.3) and the like, with Tris-borate (pH 7.5-8.3) being preferred. In addition, auxiliary components for the native polyacrylamide gel electrophoresis may be utilized in the buffer, such as EDTA. The conditions for such native polyacrylamide gel electrophoresis may include a constant power of 30 to 40 W at 4℃ to room temperature (about 20 to 25°C) for 1 hour to 4 hours.

After the native polyacrylamide gel electrophoresis, the native polyacrylamide gel is transferred onto a filter paper and contacted with X-ray film to expose the X-ray film with the radiation from the radiolabeled searching region polynucleotide. An appropriate cassette may be utilized to expose the X-ray film. The autoradiogram obtained from developing the X-ray film allows a comparison of the mobility of the radiolabeled searching region polynucleotide with the mobility of a standard. Such a mobility of the standard can be the mobility expected when the searching region polynucleotide is composed of only normal codons of the normal ERα polynucleotide. A mobility of the radiolabeled searching region polynucleotide different from the mobility of the standard typically indicates that there is one or more variant codons in the radiolabeled searching region.

The radiolabeled searching region polynucleotide may then be recovered from the native polyacrylamide gel by using heated or boiling water. The radiolabeled searching region may be PCR amplified for a second round and then prepared for sequencing. The mutation in the variant codon, if present, may then be determined similarly to the methods described above in the PCR amplification and nucleotide

sequencing methods.

The hybridization methods typically utilize a probe oligonucleotide to observe whether the probe oligonucleotide can hybridize to the searching regions. The searching regions can be provided in the hybridization methods by utilizing the searching region polynucleotide, the prepared test cDNA, the prepared test genomic DNA, a purified test ERα polynucleotide or the like. Further, the hybridization methods may restriction digest the searching region polynucleotide and then utilize the restriction digested searching region polynucleotide to observe whether the probe oligonucleotide can hybridize thereto.

The probe oligonucleotides may have a size of from 15 to 40 bp, and may have a GC content of 30% to 70%. Such probe oligonucleotides may be synthesized with a DNA synthesizer using the β-cyanoethyl phosphoamidide methods, thiophosphite methods or the like. Further, the probe oligonucleotides are typically non-radioactively labeled such as with biotin, radiolabeled such as with ^{32}P or the like.

The probe oligonucleotides may be composed of the nucleotide sequence of the searching region, when the searching region is composed of only normal codons of a normal ERα polynucleotide. Such a nucleotide sequence allows the probe oligonucleotides to hybridize to the searching region in the test ERα polynucleotide under stringent conditions, when the searching region therein is composed of only normal codons of a normal ERα polynucleotide. Examples of such probe oligonucleotides for a human test ERα polynucleotide are shown below in Table 3, in connection with the relative position of the amino acid encoded in the searching region.

Table 3

Probe oligonucleotide	relative position
SEQ ID:111, SEQ ID:112, SEQ ID:113, SEQ ID:114 or SEQ ID:115	303
SEQ ID:116, SEQ ID: 117, SEQ ID:118, SEQ ID:119 or SEQ ID:120	309
SEQ ID:121, SEQ ID: 122, SEQ ID:123, SEQ ID:124 or SEQ ID:125	390
SEQ ID:126, SEQ ID: 127, SEQ ID:128, SEQ ID:129 or SEQ ID:130	396
SEQ ID:131, SEQ ID: 132, SEQ ID:133, SEQ ID:134 or SEQ ID:135	415
SEQ ID:136, SEQ ID: 137, SEQ ID:138, SEQ ID:139 or SEQ ID:140	494
SEQ ID:141, SEQ ID: 142, SEQ ID:143, SEQ ID:144 or SEQ ID:145	531
SEQ ID:146, SEQ ID: 147, SEQ ID:148, SEQ ID:149 or SEQ ID:150	578

Typically, the hybridization methods are conducted under stringent conditions. As such stringent conditions, for example, the prehybridization or hybridization treatments are conducted in prehybridization buffer and hybridization buffer, and the washings are conducted twice for 15 minutes in washing buffer. The hybridization methods may optionally have another washing for 30 minutes in a buffer containing 0.1xSSC (0.015M NaCl, 0.0015M sodium citrate) and 0.5% SDS. As the prehybridization buffer, there may be utilized a buffer containing 6xSSC (0.9M NaCl, 0.09M sodium citrate), 5xDenhart (0.1% (w/v) phycol 400, 0.1% (w/v) polypyrolidone and 0.1% BSA), 0.5% (w/v) SDS and 100μg/ml of salmon sperm DNA. Also as the prehybridization buffer, there may be utilized a DIG EASY Hyb buffer (Boehringer Manheim) to which salmon sperm DNA is added to a concentration of 100μg/ml. Further, as the prehybridization buffer, there may be utilized a buffer containing 6xSSPE (0.9M NaCl, 0.052M NaH_2PO_4, 7.5 mM EDTA), 0.5% SDS, 5x Denhart and 0.1 mg/ml of salmon sperm DNA. As the hybridization buffer, there may be utilized the prehybridization buffer to which the probe oligonucleotide is added to a sufficient amount. The temperature of the prehybridization and hybridization treatments can vary

with the length of the probe oligonucleotide and for example, may be at the Tm value of the probe oligonucleotide to a temperature that is 2 to 3 lower than the Tm value of the probe oligonucleotide. The temperature of the washings can also vary with the length of the oligonucleotide, and for example may be conducted at room temperature. The Tm value in such cases, can be achieved by estimating the quantity of nucleotide units that should form hydrogen bonds in the hybridization buffer with the nucleotide units in the probe oligonucleotide, and then by adding the temperatures achieved from adding 2℃ for the A or T nucleotide units in the probe oligonucleotide which should form the hydrogen bond and adding 4℃ for the G or T nucleotide units in the probe oligonucleotide which should form the hydrogen bond.

For example, the hybridization methods can involve dot-blot hybridization methods, mismatch detection methods or the like.

The dot-blot hybridization methods typically involve fixing the test ERα polynucleotide to a membrane and evaluating whether the probe oligonucleotide can hybridize to the searching region in the fixed test ERα polynucleotide. In fixing test ERα polynucleotide onto the membrane, there can be utilized as the test ERα polynucleotide, the searching region polynucleotide, the prepared test cDNA, the prepared test genomic DNA, a purified test ERα polynucleotide or the like. The test ERα polynucleotide can be fixed to the membrane by incubating the test ERα polynucleotide at 90 to 100°C for 3 to 5 min, by spotting the test ERα polynucleotide onto the membrane, by drying the resulting membrane and by exposing the spotted searching region with UV light. As the membrane, there can be utilized a nylon membrane such as Hybond N (Amerscham Pharmacia). The probe oligonucleotide can then be utilized to evaluate whether the probe oligonucleotide can hybridize to the searching region. The probe oligonucleotide may be utilized by incubating the probe oligonucleotide and the test ERα polynucleotide at 40 to 50°C for 10 to 20 hours. The resulting membrane may then be washed and the hybridized probe oligonucleotide can then be detected, if present.

When the probe oligonucleotide is radiolabeled with ^{32}P, the hybridized probe oligonucleotide, if present, may be detected by exposing the resulting membrane to a X-

ray film.

When the probe oligonucleotide is nonradioactively labeled with biotin, the hybridized probe oligonucleotide, if present, may be detected with a spacer and a hybridization detection enzyme such as biotinylated alkaline phosphatase, biotinylated peroxidase or the like. When the probe oligonucleotide labeled with biotin can hybridize to the searching region, the spacer, such as streptavidin, can bind to the hybridized probe oligonucleotide labeled with biotin such that the hybridization detection enzyme can then connect to the hybridized probe oligonucleotide labeled with biotin through the spacer. The connected hybridization detection enzyme can then participate in a reaction to indicate whether the probe oligonucleotide has hybridized to the searching region in the test ERα polynucleotide. The enzymatic reaction can provide a change in color or a luminescence.

When the probe oligonucleotide does not hybridize to the searching region, it can be determined that the searching region contains one or more of the variant codons. The searching region may then be sequenced. The mutation in the variant codon, if present, may be determined similarly to the methods described above in the PCR amplification and nucleotide sequencing methods.

The mismatch detection methods are described in Biswas, I. and Hsieh, P., J. Biol. Chem., 271(9), pp. 5040-5048 (1996) as well as Nippon gene information, 1999, No. 125, Nippon Gene, Toyama. In such mismatch detection methods, a mismatch detection enzyme, such as Taq Mut S, is utilized to search certain mismatches in the hybridization of the probe oligonucleotide to the searching region. The mismatch detection enzyme allows the mismatches in the hybridization of the probe oligonucleotide with the searching region to be detected at high temperatures such as at a temperature of 75°C or lower. Typically, such mismatches in the hybridization thereof, bound with the mismatch detection enzyme, can be detected by a gel shift assay or by the dot blot hybridization methods as described in the above. When the mismatch detection enzyme can bind to a mismatched hybridization of the probe oligonucleotide and the searching region, it may be determined that the searching region contains one or more of the variant codons. The searching region may be sequenced. The mutation in

the variant codon, if present, may then be determined similarly to the methods described above in the PCR amplification and nucleotide sequencing methods.

Further, in the RFLP methods, a restriction enzyme is mixed with the test ERα polynucleotide under reacting conditions. Typically, the restriction enzyme has a restriction site overlapping with the codon in the searching region, which is suspected to be the variant codon. A successful or an unsuccessful restriction digest at the restriction site can determine whether there is the variant codon in the searching region. The results of the restriction digestion can be evaluated by gel electrophoresis analysis, such as with low melting point agarose gel electrophoresis. The searching region may then be sequenced, if needed. The mutation in the variant codon, if present, may then be determined similarly to the methods described above in the PCR amplification and nucleotide sequencing methods.

The phenotype diagnosis methods may involve searching in an amino acid sequence of the test ERα for one or more substituted amino acids which confer the activity for transactivation of the reporter gene as described in the above 5.2. After searching for the substituted amino acid, the mutation in the test ERα, if present, is determined by comparing the amino acid sequence of the test ERα to the amino acid sequence of the normal ERα. To search for the substituted amino acid in the test ERα, the searching region in the test ERα may include the amino acids in the test ERα at relative positions 303 to 578. For example, such phenotype diagnosis methods may have the searching region include an amino acid in the test ERα at one or more relative positions selected from 303, 309, 390, 396, 415, 494, 531, 578 and the like.

To search for the substituted amino acid in the test ERα, an antibody having an epitope in the searching region in the test ERα may be useful. A successful or unsuccessful binding of such an antibody can determine whether there is a substituted amino acid at the searching region in the test ERα. The mutation in the test ERα can then be determined by comparing the amino acid sequence of the test ERα with the amino acid sequence of a normal ERα.

The test ERα may be prepared from a test sample by cell extract techniques. Further, the test ERα may be prepared for the phenotype diagnosis methods by

purifying recombinant test ERα.

5.4. The reporter assay with the test ERα

A test ERα can be assayed for the activity for transactivation of the reporter gene, described in the above 5.2., by utilizing an assay cell comprising the test ERα and a chromosome which comprises the reporter gene. In such cases, the assay cell is typically exposed to a ligand and the transactivation level of the reporter gene is measured to quantitatively analyze the activity for transactivation of the reporter gene by the test ERα. Further, the activity for transactivation of the reporter gene by the test ERα can be evaluated by comparing the transactivation level of the reporter gene by the test ERα to the transactivation level of the reporter gene by a standard. Furthermore, the test ERα can be screened by selecting the test ERα in which the transactivation level of the reporter gene by the test ERα is different than the transactivation level by the standard.

The assay cell can be produced by introducing the reporter gene and a gene encoding the test ERα into a host cell. The reporter gene is inserted into a chromosome of the host cell. The test ERα gene can be introduced into the host cell for transient expression or can be introduced into the host cell so that the test ERα gene is inserted into a chromosome of the host cell. When inserting the test ERα gene into a chromosome of the host cell, the test ERα gene may be inserted into the chromosome together with the test ERα or into another chromosome in the host cell. Additionally, the reporter gene may be introduced into the host cell to produce a stably transformed cassette cell, as described in the above 5.2., and the test ERα gene may then be introduced into the stably transformed cassette cell, as described in the above 5.2.

The test ERα gene is introduced into the host cell so that the test ERα gene can be expressed in the assay cell to provide the test ERα. In this regard, such a test ERα gene typically comprises a promoter linked operably upstream from a polynucleotide which encodes the test ERα.

To introduce the test ERα gene into the host cell, conventional techniques for

introducing the test ERα gene may be applied according to the type of host cell, as described in the above 5.2. In this regard, when test ERα is introduced into the host cell for transient expression, the test ERα gene is introduced in a circular form. When inserting the test ERα gene into the chromosome of the host cell, the test ERα is introduced in a linearized form. Also, a vector may be utilized to introduce the test ERα gene or the reporter gene into the host cell, as described in the above 5.2.

Further, the test ERα gene can be introduced into the stably transformed cassette cell to provide the assay cell. In such cases, the test ERα gene may also be introduced into the stably transformed cassette cell to provide a stably transformed binary cell. Such an stably transformed binary cell has the chromosomes thereof stably comprise the test ERα gene and the reporter gene.

The host cell utilized to produce the assay cell typically fails have an expressed normal or mutant ERα. Examples of the host cells include eukaryotic cells such as HeLa cells, CV-1 cells, Hepa1 cells, NIH3T3 cells, HepG2 cells, COS1 cells, BF-2 cells, CHH-1 cells and the like.

In the reporter assay, the assay cell is typically exposed with a sufficient amount of a ligand for one to several days. Further, the ligand can be exposed to the assay cell under agonistic conditions or antagonistic conditions directed to the test ERα. The agonistic conditions typically have the assay cell exposed to the ligand as the sole agent probable of stimulating the test ERα. The antagonistic conditions typically have the assay cell exposed to the ligand and E2.

As the ligand there is usually utilized a ligand that is purely or partially antagonistic or agonistic to the normal ERα. Examples of such ligands include the partial anti-estrogens such as tamoxifen, 4-hydroxytamoxifen and raloxifene, the pure anti-estrogens such as ICI 182780 (Wakeling AE et al., Cancer Res., 512:3867-3873 (1991)) and ZM 189154 (Dukes M et al., J. Endocrinol., 141:335-341 (1994)) and the like.

After exposure, the transactivation level of the reporter gene is measured by measuring the expression level of the reporter gene. In such cases, the reporter protein or the reporter RNA (encoded by the reporter sequence) is stored in the cell or is

secreted from the cell so that the expression level can be measured therewith. The expression level of the reporter gene can be measured by a Northern blot analysis, by a Western blot analysis or by measuring the activity level of the reporter protein. The activity level of the protein typically indicates the level at which the reporter protein is expressed.

For example, when the reporter gene encodes luciferase as the reporter protein, the expression level of the reporter gene can be measured by the luminescence provided by reacting luciferin and luciferase. In such cases, a crude cell extract is produced from the cells and luciferin is added to the crude cell extract. The luciferin may be allowed to react with the luciferase in the cell extract at room temperature. The luminescence from adding luciferin is usually measured as an indicator of the expression level of the reporter gene, since the crude cell extract produces a luminescence at a strength proportional to the level of luciferase expressed in the cell and present in the crude cell extract. A luminometer may be utilized to measure the luminescence in the resulting crude cell extract.

The measured transactivation level can then be compared with a transactivation level of the reporter gene by a standard to evaluate the activity for transactivation by the test ERα. Such a transactivation level of the reporter gene by the standard in evaluating the activity for transactivation by the test ERα can be the expected transactivation level of the reporter gene in cases in which the assay cell expresses the normal ERα or an ERα which phenotype is known (instead of the test ERα). When the measured transactivation level provided by the test ERα is different than the transactivation level of the reporter gene by the standard, the test ERα may be selected as a mutant ERα.

Furthermore, mutant ligand dependent transcription factors can be screened. In such cases, the a gene encoding the test ligand dependent transcription factor, instead of the test ERα gene, in introduced into the host cell. Examples of such test ligand dependent transcription factors include a test ERβ, a test AR, a test GR, a test TR, a test PR, a test PXR, a test lipophilic vitamin receptor such a test VDR and a test RAR and the like. The reporter gene in such cases comprises an appropriate receptor responsive

sequence cognate with the provided test ligand dependent transcriptional factor, instead of the ERE.

6. Examples

6.1. Example 1 A polynucleotide encoding the human mutant ERα

6.1.1. Production of a plasmid encoding human normal ERα

A human liver cDNA library (CLONETECH, Quick clone cDNA#7113-1) is utilized to specifically PCR amplify therefrom a cDNA encoding a human normal ERα. The PCR mixture in this PCR amplification contains 10 ng of the human liver cDNA library, 10 pmol of an oligonucleotide depicted in SEQ ID:11, 10 pmol of a oligonucleotide depicted in SEQ ID:12, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). The oligonucleotides depicted in SEQ ID:11 and SEQ ID:12 are synthesized with a DNA synthesizer (Model 394, Applied Biosystems). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm that the amplified cDNA from the PCR amplification has a size of about 1.8kb. After recovering the amplified cDNA from the low melting point agarose gel, a sample of the recovered cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to reveal that the cDNA has a nucleotide sequence encoding a human normal ERα which has the amino acid sequence shown in SEQ ID:1.

Another PCR amplification is then similarly conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA. In this PCR amplification, there is utilized 100ng of the cDNA, a oligonucleotide depicted

in SEQ ID:151 and an oligonucleotide depicted in SEQ ID:12. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm that the amplified cDNA from the PCR amplification has a size of about 1.8kb. After recovering the amplified cDNA from the low melting point agarose gel, 1μg of the amplified cDNA is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the amplified cDNA. Subsequently, the resulting cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends thereof. After phenol treating the phosphorylated cDNA, the phosphorylated cDNA is ethanol precipitated to achieve a purified form of the phosphoylated cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with bacterial alkaline phosphatase (BAP) for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The restriction digested pRc/RSV is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated cDNA are used in a ligation reaction with a T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB (Luria-Bertani) medium to which ampicillin is added to a concentration of 50μg/ml (hereinafter referred to as LB-amp medium; J. Sambrook, E.F. Frisch, T. Maniatis; Molecular Cloning 2nd Edition, Cold Springs Harbor Laboratory Publishing, 1989). The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid that has a nucleotide sequence encoding human normal ERα having the amino acid sequence shown in SEQ ID:1. Such a plasmid is selected and is designated as pRc/RSV-hERαKozak.

6.1.2. Production of plasmids encoding the human mutant ERα K303R, S309F, M396V, G415V, G494V or K531E

6.1.2.1. Production of a plasmid for mutagenisis

The plasmid pRc/RSV-hERαKozak is restriction digested with restriction enzyme *Not I* for 1 hour at 37℃. The restriction digestion reaction mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm that there is DNA fragment having a size of about 1.6kb. The 1.6kb DNA fragment is then recovered from the low melting point agarose gel.

The plasmid pBluescriptII SK(+) (Stratagene) is restriction digested with *NotI* for 1 hour at 37℃ and is then treated with BAP for 1 hour at 65℃. The restriction digestion reaction mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) and the restriction digested pBluescriptII SK(+) is recovered from the low melting point agarose gel. Subsequently, 100ng of the above 1.6kb DNA fragment and 100ng of the recovered pBluescriptII SK(+) are used in a ligation reaction with T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp medium. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then restriction digested with restriction enzymes *Not I* and *Hind III*. The restriction digestion reaction mixtures are subjected to agarose gel electrophoresis. It is then confirmed that there is plasmid in which the plus strand in the plasmid contains the sense strand encoding the human normal ERα operably with M13 microphage replication origin (f1 ori). In this regard, it is confirmed that there is a plasmid which has a structure such that when the f1 ori replicates one of the strands in the plasmid, the sense strand encoding human normal ERα would be replicated therewith. Such a plasmid is selected and is designated as pSK-NN.

6.1.2.2. Site directed mutagenesis at relative positions 303, 309, 396, 415, 494 and 531

According to the methods described in McClary JA et al. (Biotechniques 1989(3): 282-289), specified mutations are introduced into the polynucleotide encoding the human normal ERα. Such procedures are described in relation with the present invention below.

The plasmid pSK-NN, provided in the above 6.1.2.1., is utilized to transform E. coli competent CJ236 cells (Takara Shuzo) according to the protocol provided with the E. coli competent CJ236 cells. A clone thereof showing ampicillin resistance is then cultured for 16 hours in a LB-amp medium. Subsequently, a colony of the clone is suspended in 10ml of a 2xYT medium to which a M13 helper phage is added to a concentration of at least $1x10^{11}$pfu/ml (hereinafter referred to as 2xYT-M13) medium. After culturing the clone in the 2xYT-M13 medium for 2 hours at 37℃, kanamycin is added thereto to a concentration of 50μg/ml and the clone is then cultured for 22 hours. The resulting suspension is centrifuged and 8ml of the resulting supernatant is transferred to a 15ml test tube. Two milliliters (2ml) of 2.5M NaCl-40% PEG8000 (Sigma) is then added to and stirred with the supernatant. The supernatant is refrigerated at 4℃ for 1 hour and is centrifuged (3,000rpm, 2,000xg, 10 minutes, 4℃) to collect the phage therefrom as a pellet. After the phage is suspended in 400μl of distilled water, an identical amount by volume of phenol is added thereto and the resulting suspension is gently shook for 5 minutes. The resulting suspension is centrifuged so that the aqueous layer therein is extracted therefrom. For a second round of phenol treatment, an identical amount by volume of phenol is then added to the aqueous layer and is vigorously shook. The resulting suspension is centrifuged so that the aqueous layer is extracted therefrom. To the aqueous layer from the second phenol treatment, an identical amount by volume of chloroform is added thereto and is vigorously shook. The resulting suspension is centrifuged (15,000rpm, 20,000x g, 5 minutes, 4℃) to extract the aqueous layer therefrom. To the aqueous layer from the chloroform treatment, there is added 800μl of 100% ethanol and 50μlof 3M sodium acetate. After refrigerating the aqueous layer therefrom at -80℃ for 20 minutes, the aqueous layer is centrifuged. The resulting pellet therefrom is rinsed with 70% ethanol

and is then dried. After pellet the residue in sterile water, the light absorbance of aqueous solution is measured at a wavelength of 260nm to calculate the amount of the single strand sense DNA encoding human normal ERα therein.

The oligonucleotides for the site directed mutagenesis are synthesized to provide the oligonucleotides depicted in SEQ ID:152, SEQ ID:153, SEQ ID:154, SEQ ID:155, SEQ ID:156 and SEQ ID:157.

In using the oligonucleotide depicted in SEQ ID:152, the AAG codon encoding the lysine present at relative position 303 is changed to an AGG codon encoding arginine.

In using the oligonucleotide depicted in SEQ ID:153, the TCC codon encoding the serine present at relative position 309 is changed to a TTC codon encoding phenylalanine.

In using the oligonucleotide depicted in SEQ ID:154, the ATG codon encoding the methionine present at relative position 396 is changed to an GTG codon encoding valine.

In using the oligonucleotide depicted in SEQ ID:155, the GGA codon encoding the glycine present at relative position 415 is changed to a GTA codon encoding valine.

In using the oligonucleotide depicted in SEQ ID:156, the GGC codon encoding the glycine present at relative position 494 is changed to a GTC codon encoding valine.

In using the oligonucleotide depicted in SEQ ID:157, the AAG codon encoding the lysine present at relative position 531 is changed to a GAG codon encoding glutamic acid.

Each of the oligonucleotides is phosphorylated with 10pmol of a polynucleotide kinase (Takara Shuzo) in the buffer provided with the polynucleotide kinase. In the phosphorylation reactions, 2mM of ATP is used in each of the reaction mixtures and the reaction mixtures are incubated at 37℃ for 30 minutes. Subsequently, about 1 pmol of the phosphoylated oligonucleotides are mixed, respectively, with 0.2 pmol of the single stand sense DNA encoding normal ERα. To produce 10μl annealing

reaction mixtures, the mixtures are then added, respectively, to annealing buffer (20 mM of Tris-Cl(pH7.4), 2 mM of $MgCl_2$, 50mM of NaCl). The annealing reaction mixtures are subjected to an incubation at 70℃ for 10 minutes, then an incubation at 37℃ for 60 minutes, which is followed by an incubation at 4℃. Synthesizing reaction mixtures are then produced therefrom by adding, respectively, to the annealing reaction mixtures, 2 units (0.25μl) of T7 DNA polymerase (New England Labs), 2 units of (0.25μl) of T4 DNA ligase (Takara Shuzo) and 1.2 μl of a synthesizing buffer (175 mM of (Tris-Cl (pH7.4), 375 mM of $MgCl_2$, 5 mM of DTT, 4 mM of dATP, 4 mM of dCTP, 4 mM of dGTP, 4mM of dTTP and 7.5 mM of ATP). The synthesizing reaction mixtures are incubated at 4℃ for 5 minutes, incubated at room temperature for 5 minutes, and then incubated at 37℃ for 2 hours, to provide synthesized DNA plasmids.

Two microliters (2μl) of each of the synthesizing reaction mixtures are then used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids from the synthesizing reactions. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

It is confirmed from the sequencing that the isolated plasmids synthesized from utilizing the oligonucleotide depicted in SEQ ID:152 provides an isolated plasmid which has in the nucleotide sequence encoding the human mutant ERα, an AGG codon corresponding to relative position 303, to provide arginine. Such an isolated plasmid is selected and is designated as pSK-NN303.

It is confirmed from the sequencing that the isolated plasmids synthesized from the oligonucleotide depicted in SEQ ID:153 provides an isolated plasmid which has in the nucleotide sequence encoding the human mutant ERα, a TTC codon corresponding to relative position 309, to provide phenylalanine. Such an isolated plasmid is selected and is designated as pSK-NN309.

It is confirmed from the sequencing that the isolated plasmids synthesized

from the oligonucleotide depicted in SEQ ID:154 provides an isolated plasmid which has in the nucleotide sequence encoding the human mutant ERα, a GTG codon corresponding to relative position 396, to provide valine. Such an isolated plasmid is selected and is designated as pSK-NN396.

It is confirmed from the sequencing that the isolated plasmids synthesized from the oligonucleotide depicted in SEQ ID:155 provides an isolated plasmid which has in the nucleotide sequence encoding the human mutant ERα, a GTA codon corresponding to relative position 415, to provide valine. Such an isolated plasmid is selected and is designated as pSK-NN415.

It is confirmed from the sequencing that the isolated plasmids synthesized from the oligonucleotide depicted in SEQ ID:156 provides an isolated plasmid which has in the nucleotide sequence encoding the human mutant ERα, a GTC codon corresponding to relative position 494, to provide valine. Such an isolated plasmid is selected and is designated as pSK-NN494.

It is confirmed from the sequencing that the isolated plasmids synthesized from the oligonucleotide depicted in SEQ ID:157 provides an isolated plasmid which has in the nucleotide sequence encoding the human mutant ERα, a GAG codon corresponding to relative position 531, to provide glutamic acid. Such as isolated plasmid is selected and is designated as pSK-NN531.

Table 4 below shows the utilized oligonucleotide for the mutagenesis, the produced plasmid therefrom and the resulting human mutant ERα therefrom.

Table 4

SEQ ID of utilized oligonucleotide	produced plasmid	human mutant ERα
SEQ ID:152	pSK-NN303	human mutant ERαK303R
SEQ ID:153	pSK-NN309	human mutant ERαS309F
SEQ ID:154	pSK-NN396	human mutant ERαM396V
SEQ ID:155	pSK-NN415	human mutant ERαG415V
SEQ ID:156	pSK-NN494	human mutant ERαG494V
SEQ ID:157	pSK-NN531	human mutant ERαK531E

The plasmids pSK-NN303, pSK-NN309, pSK-NN396, pSK-NN415, pSK-

NN494 and pSK-NN531 are each restriction digested with restriction enzyme *Not I* at 37℃ for 1 hour. Each of the restriction digestion reaction mixtures are then subjected to low melting point agarose gel electrophoresis to confirm that there are DNA fragments having a size of about 1.6kb. The 1.6kb DNA fragments are then recovered from the low melting point agarose gel.

The plasmid pRc/RSV-hERαKozak, provided in 6.1.1., is restriction digested with restriction enzyme *Not I* at 37℃ for 1 hour and is treated with BAP at 65℃ for 1 hour. The restriction digestion reaction mixture is then subjected to low melting point agarose gel electrophoresis to confirm that there is a DNA fragment having a size of about 5.5kb. The 5.5kb DNA fragment is then recovered from the low melting point agarose gel.

Subsequently, 100ng of the recovered 5.5kb DNA fragments are mixed, respectively, with 100ng of the above 1.6kb DNA fragments for a ligation reaction with T4 DNA ligase. The ligation reaction mixtures are used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp medium. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reactions. An aliquot sample of each of the isolated plasmids are then restriction digested with either restriction enzyme *Not I* or *Mlu I*. The restriction digestion reaction mixtures are then subjected to agarose gel electrophoresis. It is confirmed that there are isolated plasmids which result from the each of the restriction digestions, DNA fragments having the desired sizes. Such isolated plasmids provide DNA fragments having sizes of 5.5 and 1.6 kb in the restriction digestions with restriction enzyme *Not I* and provide DNA fragments of 7.1kb in restriction digestions with restriction enzyme *Mlu I*.

Each of the plasmids above is then PCR amplified with oligonucleotides depicted in SEQ ID:158, SEQ ID:159 and SEQ ID:160. The PCR mixtures in these PCR amplifications contain one of the plasmids, the oligonucleotide depicted in SEQ ID:158, the oligonucleotide depicted in SEQ ID:159, the oligonucleotide depicted in SEQ ID:160, 400μM of dNTPs (100μM of dATP, 100μM of dTTP, 100μM of dGTP

and 100μM of dCTP), recombinant Taq DNA polymerase (Takara Shuzo), the PCR buffer provided with the recombinant Taq DNA polymerase. In these PCR amplifications, there are repeated 30 times, an incubation cycle entailing an incubation at 94℃ for 30 seconds, then an incubation at 65℃ for 1 minute, which is followed by an incubation at 72℃ for 1 minute and 45 seconds. Ten microliters (10μl) of each of the resulting 25μl PCR mixtures are subjected to a 1% agarose gel electrophoresis (Agarose S, Nippon Gene) to confirm that the resulting plasmids have a size of about 1.2kb. The plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared samples of the plasmids are sequenced, respectively, with an ABI autosequencer (Model 377, Applied Biosystems).

It is confirmed from the sequencing that the plasmid derived from pSK-NN303 encodes the human mutant ERα K303R (AAG→AGG; lysine→arginine; relative position 303). This plasmid is designated as pRc/RSV-hERαK303R Kozak.

It is confirmed from the sequencing that the plasmid derived from pSK-NN309 encodes the human mutant ERα S309F (TCC→TTC; serine→phenylalanine; relative position 309). This plasmid is designated as pRc/RSV-hERαS309F Kozak.

It is confirmed from the sequencing that the plasmid derived from pSK-NN396 encodes the human mutant ERα M396V (ATG→GTG; methionine→valine; relative position 396). This plasmid is designated as pRc/RSV-hERαM396V Kozak.

It is confirmed from the sequencing that the plasmid derived from pSK-NN415 encodes the human mutant ERα G415V (GGA→GTA; glycine→valine; relative position 415). This plasmid is designated as pRc/RSV-hERαG415V Kozak.

It is confirmed from the sequencing that the plasmid derived from pSK-NN494 encodes the human mutant ERα G494V (GGC→GTC; glycine→valine; relative position 494). This plasmid is designated as pRc/RSV-hERαG494V Kozak.

It is confirmed from the sequencing that the plasmid derived from pSK-NN531 encodes the human mutant ERα K531E (AAG→GAG; lysine→glutamic acid; relative position 531). This plasmid is designated as pRc/RSV-hERαK531E Kozak.

Table 5 below shows the plasmid utilized to produce the plasmid and the

resulting plasmid produced therefrom.

Table 5

plasmid	produced plasmid	encoded human mutant ERα
pSK-NN303	pRc/RSV-hERK303R Kozak	human mutant ERαK303R
pSK-NN309	pRc/RSV-hERS309F Kozak	human mutant ERαS309F
pSK-NN396	pRc/RSV-hERM396V Kozak	human mutant ERαM396V
pSK-NN415	pRc/RSV-hERG415V Kozak	human mutant ERαG415V
pSK-NN494	pRc/RSV-hERG494V Kozak	human mutant ERαG494V
pSK-NN531	pRc/RSV-hERK531E Kozak	human mutant ERαK531E

6.1.3. Production of plasmids encoding the human mutant ERαG390D, S578P or G390D/S578P

6.1.3.1. Production of plasmids encoding the human mutant ERαG390D and S578P

The QuickChange Site-Directed Mutagenesis Kit (Stratagene) is used to mutagenize the plasmid pRc/RSV-hERα Kozak, described in the above 6.1.1., so that the mutagenized plasmid encodes the human mutant ERαG390D or the human mutant ERαS578P. In using the oligonucleotides depicted in SEQ ID: 17 and SEQ ID: 18, the GGT codon encoding the glycine present at relative position 390 is changed to a GAT variant codon encoding aspartic acid. In using the oligonucleotides depicted in SEQ ID:27 and SEQ ID:28, the TCC codon encoding the serine present at relative position 578 is changed to a CCC variant codon encoding proline. The manual provided with the QuickChange Site-Directed Mutagenesis Kit is used to produce the plasmids pRc/RSV-hERαG390D Kozak (GGT→GAT; glycine→asapartic acid; relative position 390) and pRc/RSV-hERαS578P Kozak (TCC→CCC; serine→proline; relative position 578). The plasmids pRc/RSV-hERαG390D Kozak and pRc/RSV-hERαS578P Kozak are sequenced to confirm that the plasmids encoding the human mutant ERα contain the desired mutation therein at relative position 390 or 578.

The QuickChange Site directed Mutagenesis Kit (Stratagene) is then used to mutagenize pRc/RSV-hERαG390D Kozak so that the mutagenized plasmid encodes the human mutant ERαG390D/S578P. The oligonucleotides depicted in SEQ ID:27 and

SEQ ID:28 are used to produce plasmid pRc/RSV-hERαG390D/S578P Kozak (GGT→GAT; glycine→asapartic acid; relative position 390 and TCC→CCC; serine→proline; relative position 578). The plasmid pRc/RSV-hERαG390D/S578P Kozak is sequenced to confirm that the plasmid encoding the human mutant ERα contains the desired mutations therein at relative positions 390 and 578.

6.1.3.2. Preparation from a test human liver tissue sample of a plasmid encoding a human mutant ERαG390D/S578P

A frozen sample of test human liver tissue was utilized to obtain a polynucleotide encoding a human mutant ERαG390D/S578P. In utilizing the test human liver tissue sample, 0.1g of the test human liver tissue sample was homogenized with a homogenizer in 5ml of a buffer containing 4M guanidium thiocyanate, 0.1M Tris-HCl (pH7.5) and 1% β mercaptoethanol. The resulting buffer was layered with 25 ml of an aqueous 5.7M CsCl solution and was ultracentrifuged at 90,000xg for 24 hours to obtain a RNA pellet. After rinsing the RNA pellet with 70% ethanol, the RNA pellet was allowed to dry at room temperature. The RNA pellet was then dissolved in 10μl of sterile water to a concentration of 1.2μg/ml. Test cDNAs were then produced by collectively using the RNAs in the RNA solution as a template in a reverse transcription reaction. In producing the test cDNAs, reverse transcriptase (Superscript II; GibcoBRL) was used with 1μl of the RNA solution, oligo-dT oligonucleotides (Amerscham Pharmacia) and the buffer provided with the reverse transcriptase. The reverse transcription reaction was allowed to react for 1 hour at 37℃, to provide the above the test cDNAs.

Similarly to the above 6.1.1., 1/50 by volume of the test cDNAs were used to produce pRc/RSV-hERαG390D/S578P Kozak. In this regard, the test cDNAs were used to specifically PCR amplify therefrom with oligonucleotides depicted in SEQ ID:11 and SEQ ID:12, the cDNA encoding the human mutant ERαG390D/S578P. The cDNA encoding the human mutant ERαG390D/S578P was then PCR amplified with the oligonucleotides depicted in SEQ ID:151 and SEQ ID:12 to add a Kozak consensus

sequence immediately upstream from the start codon (ATG) in the cDNA. The amplified product was then inserted into the *HindIII* site of the plamid pRc/RSV to provide pRc/RSV-hERαG390D/S578P Kozak.

6.2. Example 2 Production of a plasmid containing the reporter gene

An oligonucleotide depicted in SEQ ID:161 and an oligonucleotide having a nucleotide sequence complementary thereto were synthesized with a DNA synthesizer. The oligonucleotide depicted in SEQ ID: 161 was synthesized to encode one of the strands of an ERE derived from the upstream region in a Xenopus vitellogenin gene. The second oligonucleotide was synthesized to have a nucleotide sequence complementary to the oligonucleotide depicted in SEQ ID:161. The two oligonucleotides were annealed together to produce a DNA encoding an ERE (hereinafter referred to as the ERE DNA). The ERE DNA was then ligated together with a T4 DNA ligase to provide a EREx5 DNA having a 5 tandem repeat of the ERE. A T4 polynucleotide kinase was allowed to react with the EREx5 DNA to phosphorylate the ends thereof.

An oligonucleotide depicted in SEQ ID: 162 and an oligonucleotide depicted in SEQ ID: 163 were then synthesized with a DNA synthesizer. The oligonucleotide depicted in SEQ ID: 162 was synthesized to encode one of the strands in the nucleotide sequence of a TATA sequence derived from the mouse metallothionein I gene and the leader sequence thereof. The oligonucleotide depicted in SEQ ID: 163 was synthesized to encode a nucleotide sequence complementary to the oligonucleotide depicted in SEQ ID: 162. The oligonucleotides depicted in SEQ ID: 162 and SEQ ID:163 were annealed together to produce a DNA encoding the TATA sequence. A T4 polynucleotide kinase was allowed to react with the DNA encoding the TATA sequence to phosphorylate the ends thereof.

The plasmid pGL3 (Promega), which encodes the firefly luciferase gene, was restriction digested with restriction enzymes *Bgl II* and *Hind III* and was then treated with BAP at 65℃ for 1 hour. The restriction digestion reaction mixture was then subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene) to

confirm that there was a DNA fragment having the nucleotide sequence encoding the firefly luciferase. The DNA fragment having the nucleotide sequence encoding the firefly luciferase was then recovered from the low melting point agarose gel. Subsequently, 100 ng of the recovered DNA fragment and 1 μg of the DNA encoding the TATA sequence were used in a ligation reaction with T4 DNA ligase to provide a plasmid pGL3-TATA.

The plasmid pGL3-TATA was restriction digested with restriction enzyme *Sma I* and was then treated with BAP at 65℃ for 1 hour. The restriction digestion reaction mixture was then subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene) to confirm that there was a DNA fragment encoding the TATA sequence and the firefly luciferase. After recovering such a DNA fragment from the low melting point agarose gel, 100 ng of the recovered DNA fragment and 1 μg of the EREx5 DNA were used in a ligation reaction with T4 DNA ligase to provide a plasmid pGL3-TATA-EREx5.

The plasmid pUCSV-BSD (Funakoshi) was restriction digested with restriction enzyme *BamH I* to prepare a DNA encoding a blasticidin S deaminase gene expression cassette. Further, the plasmid pGL3-TATA-EREx5 was restriction digested with restriction enzyme *BamH I* and was then treated with BAP at 65℃ for 1 hour. The DNA fragment encoding a blasticidin S deaminase gene expression cassette was then mixed with the restriction digested pGL3-TATA-EREx5. The mixture was then used in a ligation reaction with T4 DNA ligase to provide plasmids. The ligation reaction mixture was used to transform E. coli competent DH5α cells. The transformed cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then restriction digested with restriction enzyme *BamH I*. The restriction digestion reaction mixtures were then subjected to agarose gel electrophoresis to confirm whether there was a plasmid which has a structure in which the DNA encoding a blasticidin S deaminase gene expression cassette has been inserted into the *Bam HI* restriction site in pGL3-TATA-EREx5. The plasmid having such a structure was selected and was

designated as pGL3-TATA-EREx5-BSD.

6.3. Example 3 Production of a stably transformed cassette cell

In order to produce stably transformed cassette cells, which stably contain in one of its chromosomes the reporter gene produced in 6.2. (hereinafter referred to as the ERE reporter gene), the plasmid pGL3-TATA-EREx5-BSD was linearized and introduced into HeLa cells.

The plasmid pGL3-TATA-EREx5-BSD was restriction digested with restriction enzyme *Sal I* to linearize pGL3-TATA-EREx5-BSD.

Approximately $5x10^5$ HeLa cells were cultured as host cells for 1 day using culture dishes having a diameter of about 10 cm (Falcon) in DMEM medium (Nissui Pharmaceutical Co.) containing 10% FBS at 37°C under the presence of 5% CO_2.

The linearized pGL3-TATA-EREx5-BSD were then introduced to the cultured HeLa cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions under the lipofection method included 5 hours of treatment, 7 μg/dish of the plasmids above and 21 μl/dish of lipofectamine.

After the lipofection treatment, the DMEM medium was exchanged with DMEM medium containing 10% FBS and the transformed HeLa cells were cultured for about 36 hours. Next, the transformed HeLa cells were removed and collected from the dish by trypsin treatment and were transferred into a container containing a medium to which blasticidin S was added to a concentration of 16 μg/ml. The transformed HeLa cells were cultured in such medium containing blasticidin S for 1 month while exchanging the medium containing blasticidin S every 3 or 4 days to a fresh batch of the medium containing blasticidin S.

The resulting clones, which were able to proliferate and produce a colony having a diameter of from 1 to several mm, were transferred as a whole to the wells of a 96-well ViewPlate (Berthold) to which medium had previously been dispensed thereto. The colonies of the clones were further cultured. When the colonies proliferated to such a degree that they covered 50% or more of the bottom surface of the well (about 5 days

after the transfer), the clones were removed and collected by trypsin treatment. The clones then were divided into 2 subcultures. One of the subcultures was transferred to a 96-well ViewPlate, which was designated as the master plate. The other subculture was transferred to a 96-well ViewPlate, which was designated as the assay plate. The master plate and the assay plate contained medium so that the clones could be cultured. The master plate was continuously cultured under similar conditions.

After culturing the subcultures in the assay plate for 2 days, the medium was then removed from the wells of the assay plate and the clones attached to the well walls were washed twice with PBS(-). A 5-fold diluted lysis buffer PGC50 (Toyo Ink) was added to the subcultures in the wells of the assay plate at 20 μl per well. The assay plate was left standing at room temperature for 30 minutes and were set on a luminometer LB96P (Berthold), which was equipped with an automatic substrate injector. Subsequently, 50 μl of the substrate solution PGL100 (Toyo Ink) was automatically dispensed to each of the lysed clones in the assay plate to measure the luciferase activity therein with the luminometer LB96P. Ten (10) clones, which exhibited a high luciferase activity were selected therefrom.

Samples of the clones in the master plate, which correspond to the selected 10 clones were then cultured at 37℃ for 1 to 2 weeks in the presence of 5% CO_2 using dishes having a diameter of about 10 cm (Falcon) in medium.

The plasmid pRc/RSV-hERαKozak was then introduced to the selected clones by a lipofection method using lipofectamine (Life Technologies) to provide a second round of clones. According with the manual provided with the lipofectamine, the conditions under the lipofection method included 5 hours of treatment, 7 μg/dish of the plasmids above and 21 μl/dish of lipofectamine. A DMSO solution containing 17β-E2 was then added to the resulting second clones to a concentration of 10nM. After culturing the second clones for 2 days, the luciferase activity was measured, similarly to the above, for each of the second clones. The clone in the master plate, which provided the second clone exhibiting the highest induction of luciferase activity, was selected as the stably transformed cassette cell which stably contained in one of its chromosomes the ERE reporter gene (hereinafter referred to as the stably transformed ERE cassette

cell).

6.4. Example 4 Production of stably transformed binary cells

Four stably transformed cells containing the ERE reporter gene with the human mutant ERαG390D, S578P or G390D/S578P or human normal ERα (hereinafter referred to as the stably transformed ERE binary cells) were produced. The first stably transformed ERE binary cell contained in its chromosomes the linearized pGL3-TATA-EREx5-BSD, which encodes the reporter gene, and the linearized pRc/RSV-hERαKozak, which encodes the human normal ERα. The second stably transformed ERE binary cell contained in its chromosomes the linearized pGL3-TATA-EREx5-BSD, which encodes the ERE reporter gene, and the linearized pRc/RSV-hERαG390D Kozak, which encodes the human mutant ERαG390D. The third stably transformed ERE binary cell contained in its chromosomes the linearized pGL3-TATA-EREx5-BSD, which encodes the ERE reporter gene, and the linearized pRc/RSV-hERαS578P Kozak, which encodes the human mutant ERαS578P. The fourth stably transformed ERE binary cell contained in its chromosomes the linearized pGL3-TATA-EREx5-BSD, which encodes the ERE reporter gene, and the linearized pRc/RSV-hERαG390D/S578P Kozak, which encodes the human mutant ERαG390D/S578P.

In order to produce the stably transformed ERE binary cells, the plasmids pGL3-TATA-EREx5-BSD, pRc/RSV-hERαG390D Kozak, pRc/RSV-hERαS578P Kozak and pRc/RSV-hERαG390D/S578P Kozak were each linearized and introduced into HeLa cells. To linearize, the plasmids above were restriction digested with restriction enzyme *Sal I*.

Approximately $5x10^5$ HeLa cells were cultured as host cells for 1 day using dishes having a diameter of about 10 cm (Falcon) in DMEM medium (Nissui Pharmaceutical Co.) containing 10% FBS at 37°C under the presence of 5% CO_2.

A linearized pGL3-TATA-EREx5-BSD was introduced, respectively, into the HeLa cells with a linearized plasmid encoding a human mutant ERα or human normal ERα, as shown in Table 6 below. The linearized plasmids were introduced into the HeLa cells by a lipofection method using lipofectamine (Life Technologies). According

with the manual provided with the lipofectamine, the conditions for each of the treatments under the lipofection method included 5 hours of treatment, 7 μg/dish of the plasmids (3.5μg each) and 21 μl/dish of lipofectamine.

Table 6

	linearized plasmids
first HeLa cells	pGL3-TATA-EREx5-BSD and pRc/RSV-hERαKozak
second HeLa cells	pGL3-TATA-EREx5-BSD and pRc/RSV-hERαG390D Kozak
third HeLa cells	pGL3-TATA-EREx5-BSD and pRc/RSV-hERαS578P Kozak
fourth HeLa cells	pGL3-TATA-EREx5-BSD and pRc/RSV-hERαG390D/S578P Kozak

After the lipofection treatment, the DMEM mediums were exchanged with DMEM medium containing 10% FBS and the transformed HeLa cells were cultured for about 36 hours. Next, the transformed HeLa cells were removed and collected, respectively, from the dishes by trypsin treatment and were transferred into a container containing a medium to which blasticidin S and G418 was added thereto. The concentration of the blasticidin S therein for each of the cell cultures was 16 μg/ml. The concentration of the G418 therein for each of the cell cultures was 800 μg/ml. The transformed HeLa cells were cultured in such medium containing blasticidin S and G418 for 1 month while exchanging the medium every 3 or 4 days to a fresh batch of the medium containing blasticidin S and G418.

The resulting clones, which were able to proliferate to a diameter of from 1 to several mm, were transferred, respectively, to the wells of 96-well ViewPlates (Berthold) to which medium had previously been dispensed thereto. The clones were further cultured. When the clones proliferated to such a degree that they covered 50% or more of the bottom surface of the well (about 5 days after the transfer), the clones were removed and collected by trypsin treatment. Each of the clones then were divided into 3 subcultures. For each of the clones, one of the subcultures was transferred to a

96-well ViewPlate, which was designated as the master plate. The other two subcultures were transferred, respectively, to 96-well ViewPlates, which were designated as the assay plates. The master plate and the assay plates contained medium so that the clones can be cultured. The master plate is continuously cultured under similar conditions. To each of the subcultures in the first assay plate, a DMSO solution containing 17β-E2 was added to a concentration of 10nM. An equivalent volume of DMSO was added to the subcultures in the second assay plate. The first and second assay plates were then cultured for 2 days.

The medium was then removed from the wells of the first and second assay plates and the clones attached to the well walls were washed twice with PBS(-). A 5-fold diluted lysis buffer PGC50 (Toyo Ink) was added to the clones in the wells of the first and second assay plates at 20 μl per well. The first and second assay plates were left standing at room temperature for 30 minutes and were set on a luminometer LB96P (Berthold), which was equipped with an automatic substrate injector. Subsequently, 50 μl of the substrate solution PGL100 (Toyo Ink) was automatically dispensed, respectively, to each of the lysed clones in the assay plates to measure the luciferase activity therein with the luminometer LB96P. Clones in master plate corresponding to the clones in the first assay plate which exhibited a 2-fold higher induction of luciferase activity (%) were then selected as the stably transformed ERE binary cell which stably contain the reporter gene with the human mutant ERαG390D, S578P or G390D/S578P gene or the human normal ERα gene.

6.5. Example 5 Reporter Assays of human mutant ERα

6.5.1 Preparation of the stably transformed ERE binary cells for the Reporter Assay

About $2x10^4$ cells of the stably transformed ERE binary cells, produced in the above 6.4., were then transferred to the wells of 96-well Luminometer plates (Corning Coaster) to culture overnight the stably transformed ERE binary cells in an E-MEM medium to which a charcoal dextran treated FBS was added to a concentration of 10%(v/v) (hereinafter referred to as charcoal dextran FBS/E-MEM medium).

6.5.2. Introduction of the plasmids encoding the human mutant ERαK303R, S309F, M396V, G415V, G494V or K531E

Seven subcultures which contained, respectively, approximately $2x10^6$ cells of the stably transformed ERE cassette cells produced in 6.3., were cultured for 1 day using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium.

For transient expression, the plasmid pRc/RSV-hERαKozak (produced in 6.1.1., encoding normal ERα) and a plasmid encoding the mutant ERα (produced in 6.1.2.2., i.e., pRc/RSV-hERαK303R Kozak, pRc/RSV-hERαS309F Kozak, pRc/RSV-hERαM396V Kozak, pRc/RSV-hERαG415V Kozak, pRc/RSV-hERαG494V Kozak or pRc/RSV-hERαK531E Kozak, each encoding a human mutant ERα) were introduced, respectively, into the subcultures of the stably transformed ERE cassette cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions for each of the treatments under the lipofection method included 5 hours of treatment, 7 μg/dish of the plasmids and 21 μl/dish of lipofectamine. After culturing the resulting cell subcultures at 37℃ for 16 hours in the presence of 5% CO_2, the charcoal dextran FBS/E-MEM medium therein was exchanged to fresh batches of the charcoal dextran FBS/E-MEM medium to further culture each of the cell subcultures for 3 hours. The cell subcultures were then collected, respectively, and uniformly suspended in charcoal dextran FBS/E-MEM medium.

6.5.3. Measurement of the activity for transactivation of the reporter gene

Four (4) general types of DMSO solutions were used to expose the cells in the subcultures prepared in the above 6.5.1. and 6.5.2. with various concentrations of a pure or partial anti-estrogen. The first DMSO solutions were prepared to contain a partial anti-estrogen (4-hydroxytamoxifen or raloxifene) at various concentrations. The second DMSO solutions were prepared to contain a pure anti-estrogen (ZM189154) at various concentrations. The third DMSO solutions were prepared to contain E2 at 10nM and a partial anti-estrogen (4-hydroxytamoxifen or raloxifene) at various concentrations. The fourth DMSO solutions were prepared to contain E2 at 10nM and a pure anti-estrogen

(ZM189154) at various concentrations.

The first, second, third or fourth DMSO solution was then added to the subcultures prepared, in the above 6.5.1. and 6.5.2., as shown in Tables 7, 8, 9 and 10 below. The first, second, third or fourth DMSO solution was added to the wells of the 96-well ViewPlates such that the concentration of the first, second, third or fourth DMSO solution in each of the wells was about 0.1% (v/v). Further, 2 controls were prepared for each of the subcultures in the wells of a 96-well ViewPlate. One of the controls was exposed to DMSO (containing no partial or pure anti-estrogen). The second control was exposed to a DMSO solution consisting essentially of 100pM of E2.

The cells were then cultured for 36 to 40 hours at 37℃ in the presence of 5% CO_2. A 5-fold diluted lysis buffer PGC50 (Toyo Ink) was added, respectively, to the cells in the wells at 50 µl per well. The 96-well ViewPlates were periodically and gently shook while being incubated at room temperature for 30 minutes. Ten microliters (10µl) of the lysed cells were then transferred, respectively, to white 96-well sample plates (Berthold) and were set on a luminometer LB96P (Berthold), which was equipped with an automatic substrate injector. Subsequently, 50 µl of the substrate solution PGL100 (Toyo Ink) was automatically dispensed, respectively, to each of the lysed cells in the white 96-well sample plates to instantaneously measure for 5 seconds the luciferase activity therein with the luminometer LB96P.

The luciferase activities resulting from the cells prepared in 6.5.2. are illustrated in Figures 1 to 32.

Figures 1 and 2 illustrate the luciferase activity provided by the human normal ERα and the human mutant ERαK303R in the presence of 4-hydroxytamoxifen or ZM189154 as the sole possible agent of stimulating the human normal ERα or the human mutant ERαK303R.

Figure 3 illustrates the luciferase activity provided by the human normal ERα and the human mutant ERαK303R in the presence of E2 with ZM189154.

Figures 4 and 5 illustrate the luciferase activity provided by the human normal ERα and the human mutant ERαS309F in the presence of 4-hydroxytamoxifen or ZM189154 as the sole possible agent of stimulating the human normal ERα or the

human mutant ERαS309F.

Figure 6 illustrates the luciferase activity provided by the human normal ERα and the human mutant ERαS309F in the presence of E2 with ZM189154.

Figures 7 and 8 illustrate the luciferase activities provided by the human normal ERα and the human mutant ERαM396V in the presence of 4-hydroxytamoxifen or raloxifene as the sole possible agent of stimulating the human normal ERα or the human mutant ERαM396V.

Figures 9 to 11 illustrate the luciferase activity provided by the human normal ERα and the human mutant ERαM396V in the presence of E2 with 4-hydroxytamoxifen, raloxifene or ZM189154.

Figures 12 and 13 illustrate the luciferase activity provided by the human normal ERα and the human mutant ERαG415V in the presence of 4-hydroxytamoxifen or ZM189154 as the sole possible agent of stimulating the human normal ERα or the human mutant ERαG415V.

Figures 14 and 15 illustrate the luciferase activity provided by the human normal ERα and the human mutant ERαG415V in the presence of E2 with 4-hydroxytamoxifen or ZM189154.

Figures 16 to 17 illustrate the luciferase activity provided by the human normal ERα and the human mutant ERαG494V in the presence of 4-hydroxytamoxifen or raloxifene as the sole possible agent of stimulating the human normal ERα or the human mutant ERαG494V.

Figures 18 to 20 illustrate the luciferase activity provided by the human normal ERα and the human mutant ERαG494V in the presence of E2 with 4-hydroxytamoxifen, raloxifene or ZM189154.

Figures 21 to 26 illustrate the luciferase activity provided by the human normal ERα and the human mutant ERαK531E in the presence of 4-hydroxytamoxifen, raloxifene or ZM189154 as the sole possible agent of stimulating the human normal ERα or the human mutant ERαK531E.

Figures 27 to 32 illustrate the luciferase activity provided by the human normal

ERα and the human mutant ERαK531E in the presence of E2 with 4-hydroxytamoxifen, raloxifene or ZM189154.

The luciferase activities resulting from the cells prepared in 6.5.1. are illustrated in Figures 33 to 48.

Figures 33 to 40 illustrate the luciferase activity provided by the human normal ERα, human mutant ERαG390D, human mutant ERαS578P and human mutant ERαG390D/S578P in the presence of 4-hydroxytamoxifen, raloxifene or ZM189154 as the sole probable agent of stimulating the human normal ERα, human mutant ERαG390D, human mutant ERαS578P and human mutant ERαG390D/S578P.

Figures 41 to 48 illustrate the luciferase activity provided by the human normal ERα, human mutant ERαG390D, human mutant ERαS578P and human mutant ERαG390D/S578P in the presence of E2 with 4-hydroxytamoxifen, raloxifene or ZM189154.

Table 7

	utilized plasmid for human normal or mutant ERα	DMSO solution	exposed partial or pure anti-estrogen
1	pRC/RSV-hERαKozak	first	4-hydroxytamoxifen
2	pRC/RSV-hERαK303R Kozak	first	4-hydroxytamoxifen
3	pRC/RSV-hERαKozak	second	ZM189154
4	pRC/RSV-hERαK303R Kozak	second	ZM189154
5	pRC/RSV-hERαKozak	fourth	ZM189154
6	pRC/RSV-hERαK303R Kozak	fourth	ZM189154
7	pRC/RSV-hERαKozak	first	4-hydroxytamoxifen
8	pRC/RSV-hERαS309F Kozak	first	4-hydroxytamoxifen
9	pRC/RSV-hERαKozak	second	ZM189154
10	pRC/RSV-hERαS309F Kozak	second	ZM189154
11	pRC/RSV-hERαKozak	fourth	ZM189154
12	pRC/RSV-hERαS309F Kozak	fourth	ZM189154

Table 8

	utilized plasmid for human normal or mutant ERα	DMSO solution	exposed partial or pure anti-estrogen
13	pRC/RSV-hERαKozak	first	4-hydroxytamoxifen
14	pRC/RSV-hERαM396V Kozak	first	4-hydroxytamoxifen
15	pRC/RSV-hERαKozak	first	raloxifene
16	pRC/RSV-hERαM396V Kozak	first	raloxifene
17	pRC/RSV-hERαKozak	third	4-hydroxytamoxifen
18	pRC/RSV-hERαM396V Kozak	third	4-hydroxytamoxifen
19	pRC/RSV-hERαKozak	third	raloxifene
20	pRC/RSV-hERαM396V Kozak	third	raloxifene
21	pRC/RSV-hERαKozak	fourth	ZM189154
22	pRC/RSV-hERαM396V Kozak	fourth	ZM189154
23	pRC/RSV-hERαKozak	first	4-hydroxytamoxifen
24	pRC/RSV-hERαG415V Kozak	first	4-hydroxytamoxifen
25	pRC/RSV-hERαKozak	second	ZM189154
26	pRC/RSV-hERαG415V Kozak	second	ZM189154
27	pRC/RSV-hERαKozak	third	4-hydroxytamoxifen
28	pRC/RSV-hERαG415V Kozak	third	4-hydroxytamoxifen
29	pRC/RSV-hERαKozak	fourth	ZM189154
30	pRC/RSV-hERαG415V Kozak	fourth	ZM189154

Table 9

	utilized plasmid for human normal or mutant ERα	DMSO solution	exposed partial or pure anti-estrogen
31	pRC/RSV-hERαKozak	first	4-hydroxytamoxifen
32	pRC/RSV-hERαG494V Kozak	first	4-hydroxytamoxifen
33	pRC/RSV-hERαKozak	first	raloxifene
34	pRC/RSV-hERαG494V Kozak	first	raloxifene
35	pRC/RSV-hERαKozak	third	4-hydroxytamoxifen
36	pRC/RSV-hERαG494V Kozak	third	4-hydroxytamoxifen
37	pRC/RSV-hERαKozak	third	raloxifene
38	pRC/RSV-hERαG494V Kozak	third	raloxifene
39	pRC/RSV-hERαKozak	fourth	ZM189154
40	pRC/RSV-hERαG494V Kozak	fourth	ZM189154
41	pRC/RSV-hERαKozak	first	4-hydroxytamoxifen
42	pRC/RSV-hERαK531E Kozak	first	4-hydroxytamoxifen
43	pRC/RSV-hERαKozak	first	raloxifene
44	pRC/RSV-hERαK531E Kozak	first	raloxifene
45	pRC/RSV-hERαKozak	second	ZM189154
46	pRC/RSV-hERαK531E Kozak	second	ZM189154
47	pRC/RSV-hERαKozak	third	4-hydroxytamoxifen
48	pRC/RSV-hERαK531E Kozak	third	4-hydroxytamoxifen
49	pRC/RSV-hERαKozak	third	raloxifene
50	pRC/RSV-hERαK531E Kozak	third	raloxifene
51	pRC/RSV-hERαKozak	fourth	ZM189154
52	pRC/RSV-hERαK531E Kozak	fourth	ZM189154

Table 10

	human normal or mutant ERα encoded in the chromosomes	DMSO solution	exposed partial or pure anti-estrogen
53	human normal ERα	first	4-hydroxytamoxifen
54	human mutant ERαG390D	first	4-hydroxytamoxifen
55	human mutant ERαS578P	first	4-hydroxytamoxifen
56	human mutant ERαG390D/S578P	first	4-hydroxytamoxifen
57	human normal ERα	first	raloxifene
58	human mutant ERαG390D/S578P	first	raloxifene
59	human normal ERα	second	ZM189154
60	human mutant ERαG390D/S578P	second	ZM189154
61	human normal ERα	third	4-hydroxytamoxifen
62	human mutant ERαG390D	third	4-hydroxytamoxifen
63	human mutant ERαS578P	third	4-hydroxytamoxifen
64	human mutant ERαG390D/S578P	third	4-hydroxytamoxifen
65	human normal ERα	third	raloxifene
66	human mutant ERαG390D/S578P	third	raloxifene
67	human normal ERα	fourth	ZM189154
68	human mutant ERαG390D/S578P	fourth	ZM189154

6.6. Example 6 Comparative dually transient reporter assay

Approximately $2x10^6$ HeLa cells were cultured for 1 day using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium at 37°C in the presence of 5% CO_2. After culturing the HeLa cells, the HeLa cells were divided into two subcultures.

Subsequently, 3.75 μg of pRc/RSV-hERαKozak and 3.75 μg of pGL3-TATA-EREx5 were introduced into the HeLa cells in the first subculture by a lipofection method using lipofectamine for transient expression. In the second subculture, 3.75 μg of pRc/RSV-hERαK531E Kozak and 3.75 μg of pGL3-TATA-EREx5 were introduced by the lipofection method using lipofectamine for transient expression. The first and second subcultures were then cultured at 37°C for 16 hours in the presence of 5% CO_2. After exchanging the charcoal dextran FBS/E-MEM medium therein with a fresh batch

of charcoal dextran FBS/E-MEM medium the first and second subcultures were then similarly cultured for 3 hours. The cells in the first and second subcultures were then collected, respectively, and were uniformly suspended in charcoal dextran FBS/E-MEM medium.

Two (2) general types of DMSO solutions were prepared to expose the cells in the first and second subcultures. The first DMSO solutions were prepared to contain 4-hydroxytamoxifen at various concentrations. The second DMSO solutions were prepared to contain 10nM of E2 and 4-hydroxytamoxifen at various concentrations.

The first and second DMSO solutions were then mixed, respectively, with the first and second subcultures in 96-well ViewPlates such that the concentration of the first or second DMSO solution in each of the wells was about 0.1% (v/v).

The first and second subcultures were then cultured for 36 hours at 37℃ in the presence of 5% CO_2. A 5-fold diluted lysis buffer PGC50 (Nippon Gene) was added, respectively, to the first and second subcultures in the wells at 50 μl per well. The 96-well ViewPlates were periodically and gently shook while being incubated at room temperature for 30 minutes. Ten microliters (10μl) of the resulting lysed cells were then transferred, respectively, to white 96-well sample plates (Berthold) and were set on a luminometer LB96P (Berthold), which was equipped with an automatic substrate injector. Subsequently, 50 μl of the substrate solution PGL100 (Toyo Ink) was automatically dispensed, respectively, to each of the lysed cells in the white 96-well sample plates to instantaneously measure for 5 seconds the luciferase activity therein with the luminometer LB96P.

The luciferase activity from the dually transient reporter assay are shown in Figures 49 to 52.

Figures 49 and 50 illustrate the luciferase activity provided by the human normal ERα and human mutant ERαK531E in the presence of 4-hydroxytamoxifen as the sole probable agent of stimulating the human normal ERα or human mutant ERαK531E.

Figures 51 and 52 illustrate, respectively, the luciferase activity of mutant human normal ERα and human mutant ERαK531E in the presence of E2 with 4-

hydroxytamoxifen.

6.7. Example 7 Search oligonucleotides

In order to search for a variant codon that encodes a substituted amino acid in a human test ERα, search oligonucleotides are designed so that the search oligonucleotides can anneal to a searching region in a human test ERα gene when the human test ERα gene encodes a human normal ERα. The searching regions include the codon encoding the amino acid at relative position 303, the codon encoding the amino acid at relative position 309, the codon encoding the amino acid at relative position 390, the codon encoding the amino acid at relative position 396, the codon encoding the amino acid at relative position 415, the codon encoding the amino acid at relative position 494, the codon encoding the amino acid at relative position 531 or the codon encoding the amino acid at relative position 578. Further the oligonucleotides are designed to have a GC content of from 30% to 70% and a size of 20bp. Based on the oligonuleotides so designed, the oligonucleotides of the present invention indicated by the above are synthesized with a DNA synthesizer (Model 394, Applied Biosystems).

6.8 Example 8 Genotype diagnosis by PCR amplification and nucleotide sequencing methods

A test human liver tissue sample is used to diagnose the genotype of the test ERα polynucleotide therein. In utilizing the test human liver tissue sample, 0.1g of the test human liver tissue sample is homogenized with a homogenizer in 5ml of a buffer containing 4M guanidium thiocyanate, 0.1M Tris-HCl (pH7.5) and 1% β mercaptoethanol. The resulting buffer is layered with 25 ml of an aqueous 5.7M CsCl solution and is ultracentrifuged at 90,000xg for 24 hours to obtain a RNA pellet. After rinsing the RNA pellet with 70% ethanol, the RNA pellet is allowed to air dry at room temperature. The RNA pellet is then dissolved in 10μl of sterile water to a concentration of 1.2μg/ml. A solution of test cDNAs is then produced by collectively using the RNAs in the RNA solution as a template in a reverse transcription reaction. In producing the test cDNAs, Superscript II (Gibco) was used with 1μl of the RNA

solution, oligo-dT oligonucleotides (Amerscham-Pharmacia) and the buffer provided with the oligo-dT oligonucleotides. The reverse transcription reaction was allowed to react for 1 hour at 37℃.

Using 1/50 by volume samples of the test cDNAs, a PCR amplification is conducted with combinations of the search oligonucleotides shown in Table 11 below.

Table 11

	Search Oligonucleotides
1	SEQ ID:32 and SEQ ID:38
2	SEQ ID:42 and SEQ ID:48
3	SEQ ID:52 and SEQ ID 58
4	SEQ ID:62 and SEQ ID:68
5	SEQ ID:72 and SEQ ID:78
6	SEQ ID:82 and SEQ ID:88
7	SEQ ID:92 and SEQ ID:98
8	SEQ ID:109 and SEQ ID: 110

The PCR mixtures in these PCR amplifications contain the test cDNAs, AmpliTaq DNA polymerase (Perkin Elmer), 100μM of dNTPs (dATP, dTTP, dGTP, dCTP), one of the combinations of the search oligonucleotides and the buffer provided with the AmpliTaq Polymerase. In this PCR amplification, there are repeated 35 times for each of the PCR amplifications, an incubation cycle entailing an incubation at 95℃ for 1 minute, then an incubation at 55°C for 30 sec, which is followed by an incubation at 72°C for 1 minute. The obtained searching region polynucleotides are subjected to 1% low melting point agarose gel electrophoresis (Agarose L, Nippon Gene) and are recovered. Using whole amounts of the recovered searching region polynucleotides, the searching regions are sequenced. The nucleotide sequences of the searching regions are compared to the nucleotide sequence encoding human normal ERα.

6.9. Example 9 Genotype diagnosis by SSCP methods

6.9.1. Extraction of test genomic DNAs from a test tissue sample

Test genomic DNAs from a test tissue sample is prepared by the methods described in TAKARA PCR Technical news No.2, Takara Shuzo (September 1991).

This procedure in relation with the present invention is described below.

Two (2) to 3 hair samples from a test subject are washed with sterile water and then 100% ethanol. After air drying the hair samples, the hair samples are cut to 2 to 3 mm and are transferred to a plastic test tube. Two hundred microliters (200µl) of BCL buffer (10mM Tris-HCl (pH.7.5), 5mM $MgCl_2$, 0.32M sucrose, 1% Triton X-100) are added thereto. Subsequently, a Proteinase K solution and a SDS solution are mixed therewith to amount to 100 µg/ml and 0.5% (w/v), respectively.

After incubating the resulting mixture for 1 hour at 70℃, the mixture is phenol-chloroform extracted to recover the aqueous layer therefrom. In the phenol-chloroform extraction, a substantially equal volume of phenol-chloroform is added to the mixture. The mixture is shaken vigorously and is centrifuged (15,000 rpm, 20,000xg, 5 min, 4°C). The aqueous layer therefrom is extracted with a pipette so that the phenol layer is not disturbed. A second phenol-chloroform extraction is then similarly conducted with the aqueous layer.

A substantially equal volume of chloroform is mixed with the aqueous layer from the second phenol-chloroform extraction, to extract the aqueous layer from the resulting chloroform mixture. In this extraction with chloroform, the chloroform mixture is shaken vigorously and is centrifuged, so that the aqueous layer can be extracted from the chloroform mixture. Five hundred microliters (500 µl) of 100% ethanol is then added to the aqueous layer from the chloroform mixture. The test genomic DNAs therein is precipitated at -80°C for 20 minutes and is then centrifuged to obtain a pellet of the test genomic DNAs. The obtained pellet of the test genomic DNAs is dried and dissolved in sterile water, to so that test genomic DNAs can provide a test ERα polynucleotide.

Alternatively, peripheral blood can be used as a test sample from which test genomic DNAs can be obtained. Ten milliliters (10ml) of blood is collected from a test subject and test genomic DNAs are extracted from the blood, using a DNA Extraction kit (Stratagene).

6.9.2. Analysis of test genomic DNA by the PCR-SSCP method

Combinations of a forward search oligonucleotide and a reverse search oligonucleotide are selected for PCR amplifications with the test genomic DNAs. The combinations of the forward and reverse search oligonucleotide are selected, based on the locus of the searching regions in the test ERα polynucleotide. The combinations of the forward and reverse search oligonucleotides in connection with the searching regions which are suspected to contain the variant codon encoding a substituted amino acid at the provided relative positions are shown in Table 12 below.

Table 12

searching region	Forward search oligonucleotide	Reverse search oligonucleotide
relative position 303	SEQ ID:29, SEQ ID:30, SEQ ID:31, SEQ ID:32 or SEQ ID:33	SEQ ID:34, SEQ ID:35, SEQ ID:36, SEQ ID:37 or SEQ ID:38
relative position 309	SEQ ID:39, SEQ ID:40, SEQ ID:41, SEQ ID:42 or SEQ ID:43	SEQ ID:44, SEQ ID:45, SEQ ID:46, SEQ ID:47 or SEQ ID:48
relative position 390	SEQ ID:49, SEQ ID:50, SEQ ID:51, SEQ ID:52 or SEQ ID:53	SEQ ID:54, SEQ ID:55, SEQ ID:56, SEQ ID:57 or SEQ ID:58
relative position 396	SEQ ID:59, SEQ ID:60, SEQ ID:61, SEQ ID:62 or SEQ ID:63	SEQ ID:64, SEQ ID:65, SEQ ID:66, SEQ ID:67 or SEQ ID:68
relative position 415	SEQ ID:69, SEQ ID:70, SEQ ID:71, SEQ ID:72 or SEQ ID:73	SEQ ID:74, SEQ ID:75, SEQ ID:76, SEQ ID:77 or SEQ ID:78
relative position 494	SEQ ID:79, SEQ ID:80, SEQ ID:81, SEQ ID:82 or SEQ ID:83	SEQ ID:84, SEQ ID:85, SEQ ID:86, SEQ ID:87 or SEQ ID:88
relative position 531	SEQ ID:89, SEQ ID:90, SEQ ID:91, SEQ ID:92 or SEQ ID:93	SEQ ID:94, SEQ ID:95, SEQ ID:96, SEQ ID:97 or SEQ ID:98
relative position 578	SEQ ID:99, SEQ ID:100, SEQ ID:101, SEQ ID:102 or SEQ ID:103	SEQ ID:104, SEQ ID:105, SEQ ID:106, SEQ ID:107 or SEQ ID:108

The combinations of the forward and reverse search oligonucleotides are synthesized with a DNA synthesizer. Each of the forward and reverse search oligonucleotides are labeled with ^{32}P using a DNA MEGALABEL kit (Takara Shuzo). The test genomic DNAs are then used, respectively, in the PCR amplifications to provide amplified searching region polynucleotides. Each of the PCR mixtures in these PCR amplifications contain Amplitaq DNA Polymerase (Perkin Elmer), 400μM of dNTPs (100μM of dATP, 100μM of dTTP, 100μM of dGTP and 100μM of dCTP), 100 pmol of the ^{32}P labeled forward search oligonucleotide, 100 pmol of the ^{32}P labeled reverse search oligonucleotide, 1μg of the test genomic DNA and the buffer provided

with the Amplitaq DNA Polymerase. In each of these PCR amplifications, there are repeated 35 times for each of the PCR amplifications, an incubation cycle entailing an incubation at 94°C for 1 minute, then an incubation at 55°C for 30 seconds, which is followed by an incubation at 72°C for 1 minute.

After the PCR amplifications, 1/20 by volume samples from each of the amplified searching region polynucleotides are heat denatured in 80% formamide at 80°C for 5 minutes. Subsequently, each the heat denatured searching region polynucleotides are subjected to electrophoresis in 5% native polyacrylamide gels using 180 mM Tris-borate buffer (pH 8.0). The conditions for electrophoresis include a room temperature air cooling and a constant power of 40W for 60 min. After electrophoresis, the 5% native polyacrylamide gels are autoradiographed using X-ray films by using conventional procedures to detect the radioactivity of the searching regions.

Since a product encoding the variant codon has a different mobility in the 5% native polyacrylamide gel as compared with a product encoding a normal codon, a comparison of each of the mobilities of the searching region polynucleotides with a standard polynucleotide encoding a corresponding region in a human normal ERα detects the presence or absence of a mutation in the searching regions.

6.9.3. Determination of mutation

After detecting a variant codon in the searching regions, 1mm square portions containing the searching region polynucleotides are cut out of the 5% native polyacrylamide gels. Each of the 1mm square portions are treated at 90°C for 10 min in 100 μl of sterile water to recover the searching region polynucleotides from the 1mm square portions. Subsequently, 1/20 by volume samples of the searching region polynucleotides are then used, respectively, in a second round of PCR amplifications. The oligonucleotides in these PCR amplifications used the combinations of the search oligonucleotides used in the above 6.9.2. Each of the PCR mixtures in these PCR amplifications contain Amplitaq DNA Polymerase (ABI), 400μM of dNTPs (100μM of dATP, 100μM of dTTP, 100μM of dGTP and 100μM of dCTP), the forward search oligonucleotide, the reverse search oligonucleotide, one of the test DNA fragments and

the buffer provided with the Amplitaq DNA polymerase. In each of these PCR amplifications, there are repeated 35 times, an incubation cycle entailing an incubation at 94°C for 1 minute, then an incubation at 55°C for 30 seconds, which is followed by an incubation at 72°C for 1 minute.

After completion of the reaction, the amplified searching region polynucleotides are subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene). After recovering the amplified searching region polynucleotides from the low melting point agarose gels, the recovered searching region polynucleotides are prepared with a Dye Terminator cycle sequence ready reaction kit (Applied Biosystems). The prepared sample of the searching region polynucleotides are sequenced, respectively, with an ABI autosequencer (Model 377, Applied Biosystems), to determine the mutation in the variant codons, if present, in the searching regions.

6.10. Example 10 Genotype diagnosis by RFLP methods

Combinations of a forward search oligonucleotide and a reverse search oligonucleotide are selected for PCR amplifications with test genomic DNAs or a test cDNAs. The combinations of the forward and reverse search oligonucleotides are selected, based on the locus of the searching regions in the test ERα polynucleotide. The combinations of the forward and reverse search oligonucleotides are shown in Table 13 below, in connection with the searching regions which are suspected to contain a variant codon encoding a substituted amino acid at the provided relative positions in Table 13 below.

Table 13

Searching region	oligonucleotides
Relative position 303	SEQ ID:164 and SEQ ID:165
Relative position 309	SEQ ID:166 and SEQ ID:167
Relative position 396	SEQ ID:168 and SEQ ID:169
Relative position 415	SEQ ID:170 and SEQ ID:171
Relative position 494	SEQ ID:172 and SEQ ID:173
Relative position 531	SEQ ID:174 and SEQ ID:175

The test genomic DNAs or test cDNAs are used in the PCR amplifications to provide amplified searching region polynucleotides having a size of about 100 or 160 bp. Each of the PCR mixtures in these PCR amplifications contain Amplitaq DNA Polymerase (ABI), the test genomic DNAs or test cDNAs, dNTPs (dATP, dTTP, dGTP and dCTP), the forward search oligonucleotide, the reverse search oligonucleotide and the buffer provided with the Amplitaq DNA Polymerase. In each of these PCR amplifications, there are repeated 35 times, an incubation cycle entailing an incubation at 94°C for 1 minute, then an incubation at 55°C for 30 seconds, which is followed by an incubation at 72°C for 1 minute.

Samples of each of the searching region polynucleotides are then mixed, respectively, with various restriction enzymes for restriction digestion reactions (one restriction enzyme per restriction digestion reaction) and are incubated at 37℃ at 1 hour. The restriction digestion reaction mixtures are subjected to agarose gel electrophoresis to confirm whether the searching region polynucleotides are successfully restriction digested with one of the various restriction enzymes. A successful restriction digest with the restriction enzymes shown in Table 14 and Table 15 below indicate whether there is in the searching region, a variant codon encoding a substituted amino acid at the provided relative position in Table 14 and Table 15 below

Table 14

	relative position 309	relative position 494
restriction enzyme	*Apa I*	*Stu I*
approximate length of searching region	100 bp	150 bp
when encoding normal codon		
restriction digestion	yes	yes
approximate length of resulting DNA fragments	40 bp/60 bp	100 bp/50 bp

In reference to Table 14, an unsuccessful restriction digestion with the provided restriction enzyme at the codon encoding the amino acid at the provided relative position, indicates that such a codon is a variant codon. In such cases, the searching regions are sequenced with an ABI autosequencer (Model 377, Applied Biosystems) to determine the mutation in the variant codons, if present, in the searching regions.

Table 15

	relative position 303	relative position 396	relative position 415	relative position 531
restriction enzyme:	*Stu I*	*ApaL I*	*Kpn I*	*Sac I*
approximate length of searching region:	100 bp	100 bp	100 bp	100 bp
when encoding normal codon				
restriction digestion:	no	no	no	no
approximate length of resulting DNA fragments:	–	–	–	–
when encoding variant codon				
when variant codon sequence is:	AGG	GTG	GTA	GAG
restriction digestion:	yes	yes	yes	yes
approximate length of resulting DNA fragments:	40 bo/60 bp	40 bo/60 bp	40 bo/60 bp	40 bo/60 bp

In reference to Table 15, a successful restriction digestion with the provided restriction enzyme at the codon encoding the amino acid at the provided relative position, indicates that such a codon is a variant codon. In such cases, it is determined that the mutations in the variant codons, if present, are the nucleotide sequences provided in the above Table 15.

6.11. Example 11 Genotype diagnosis by southern hybridization methods

Five micrograms (5μg) of test genomic DNA, provided in 6.9.1., is thoroughly restriction digested with the restriction enzyme *Stu I*. The restriction digestion reaction mixture is subjected to electrophoresis at 20V for 16 hours with a 4% Nusieve 3:1 agarose gel (FMC BIO). The capillary alkali blotting method (Hybond blotting membrane manual, Amerscham) is used to blot for 2 hours a nylon membrane with the separated DNA fragments in the 4% Nuseive 3:1 agarose gel to the nylon membrane. Followed by lightly washing the blotted filter with 2xSSC buffer (0.3M NaCl, 0.33M Na-Citrate, pH 7.0), the blotted nylon membrane is dried at 80℃ for 90 minutes.

The blotted nylon membrane is treated at 55℃ for 16 hours with prehybridization buffer (6xSSPE (0.9M NaCl, 0.052M NaH_2PO_4, 7.5 mM EDTA), 0.5% SDS, 5x Denhart and 0.1 mg/ml of salmon sperm DNA). The prehybridization buffer is then exchanged with an equal volume of hybridization buffer (6xSSPE (0.9M NaCl, 0.052M NaH_2PO_4, 7.5 mM EDTA), 0.5% SDS, 5x Denhart, 0.1 mg/ml of salmon sperm DNA and a ^{32}P labeled probe oligonucleotide). In the hybridization buffer, the radioactive concentration of the ^{32}P labeled probe oligonucleotide is at least $10x10^8$ cpm for every 150ml of the hybridization buffer. As the ^{32}P labeled probe oligonucleotide, there is utilized the oligonucleotide depicted in SEQ ID:81 which is labeled with ^{32}P at the ends thereof. The ^{32}P labeled probe is produced by incubating at 37℃ for 1 hour with γ ^{32}P-ATP, T4 polynucleotide kinase and 1 μg of the oligonucleotide depicted in SEQ ID:81 in the buffer provided with the T4 polynucleotide kinase.

After the hybridization, the blotted nylon membrane is washed twice with washing buffer containing 1xSSC (0.15 M NaCl, 15 mM sodium citrate) and 0.5% SDS.

In washing the blotting filter twice, the blotted nylon membrane is incubated after each washing at 62℃ for 40 minutes in the washing buffer.

The blotted membrane is then autoradiographed for 10 days with x-ray film to analyze whether the restriction enzyme *Stu I* is successful in restriction digesting at the restriction site therein overlapping with the codon in the searching region which is suspected to be a variant codon encoding a substituted amino acid at relative position 494. A successful restriction digest with the restriction enzyme *Stu I* indicates that there is in the test ERα polynucleotide, a nucleotide sequence encompassing AGGCCT, overlapping with the codon encoding the amino acid at relative position 494. In such cases, it is determined that the test ERα is a normal ERα. An unsuccessful restriction digest with the restriction enzyme *Stu I* at the corresponding locus, indicates that there is in the test ERα polynucleotide, a variant codon encoding a substituted amino acid at relative position 494. In such cases, the searching region is sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to determine the mutation in the variant codon, if present, in the searching region.

6.12. Example 12 Production of a plasmid encoding human normal AR

A human prostate cDNA library (CLONETECH, Quick clone cDNA#7123-1) is utilized to PCR amplify therefrom a cDNA encoding a human normal AR (Genbank Accession No. M23263). The PCR mixture in this PCR amplification contains 10 ng of the human prostate cDNA library, 10 pmol of an oligonucleotide depicted in SEQ ID:176, 10 pmol of an oligonucleotide depicted in SEQ ID:177, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). The oligonucleotides depicted in SEQ ID:176 and SEQ ID:177 are synthesized with a DNA synthesizer (Model 394, Applied Biosystems,). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm with ethidium bromide staining,

that the cDNA encoding the human normal AR is PCR amplified. After recovering the amplified cDNA from the low melting point agarose gel, a sample of the recovered cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

Another PCR amplification is then conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA. The PCR mixture in this PCR amplification contains 100ng of the cDNA encoding the human normal AR, an oligonucleotide depicted in SEQ ID:178 and an oligonucleotide depicted in SEQ ID:179, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). In this PCR amplification, there is repeated 25 times an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene). After recovering the amplified cDNA from the low melting point agarose gel, 1μg of the amplified cDNA is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the amplified cDNA. Subsequently, the resulting cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends thereof. After phenol treating the phosphorylated cDNA, the phosphorylated cDNA is ethanol precipitated to achieve a purified form of the phosphoylated cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with BAP for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The restriction digested pRc/RSV is then treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated cDNA are used in a ligation reaction with a T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are

cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids is then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid encoding the human normal AR. Such a plasmid is selected and is designated as pRc/RSV-hAR Kozak.

6.13. Example 13 Production of a plasmid encoding a human normal GR

A human liver cDNA library (CLONETECH, Quick clone cDNA#7113-1) is utilized to PCR amplify therefrom a cDNA encoding a normal GR (Genbank Accession No. M10901). The PCR mixture in this PCR amplification contains 10 ng of the human liver cDNA library, 10 pmol of an oligonucleotide depicted in SEQ ID:180, 10 pmol of an oligonucleotide depicted in SEQ ID:181, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). The oligonucleotides depicted in SEQ ID:180 and SEQ ID:181 are synthesized with a DNA synthesizer (Model 394, Applied Biosystems). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 60℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm with ethidium bromide staining, that the cDNA encoding the human normal GR is PCR amplified. After recovering the amplified cDNA from the low melting point agarose gel, a sample of the recovered cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

Another PCR amplification is then conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA. The PCR mixture in this PCR amplification contains 100ng of the cDNA encoding the human

normal GR, an oligonucleotide depicted in SEQ ID:182 and an oligonucleotide depicted in SEQ ID:183, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). In this PCR amplification, there is repeated 25 times an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 60℃ for 3 minutes. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene). After recovering the amplified cDNA from the low melting point agarose gel, 1μg of the amplified cDNA is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the amplified cDNA. Subsequently, the resulting cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends of the cDNA. After phenol treating the phosphorylated cDNA, the phosphorylated cDNA is ethanol precipitated to achieve a purified form of the phosphoylated cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with BAP for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The restriction digested pRc/RSV is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated cDNA are used in a ligation reaction with a T4 DNA ligase. The reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid encoding the normal GR. The plasmid is selected and is designated as pRc/RSV-hGR Kozak.

6.14. Example 14 Production of a plasmid encoding a human normal PR

A human liver cDNA library (CLONETECH, Quick clone cDNA#7113-1) is utilized to PCR amplify therefrom a cDNA encoding a normal PR (Genbank Accession No. M15716). The PCR mixture in this PCR amplification contains 10 ng of the human liver cDNA library, 10 pmol of a oligonucleotide depicted in SEQ ID:184, 10 pmol of a oligonucleotide depicted in SEQ ID:185, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). The oligonucleotides depicted in SEQ ID:184 and SEQ ID:185 are synthesized with a DNA synthesizer (Model 394, Applied Biosystems). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 55℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm with ethidium bromide staining, that the cDNA encoding human normal PR is PCR amplified. After recovering the amplified cDNA from the low melting point agarose gel, a sample of the recovered cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

Another PCR amplification is then conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA. The PCR mixture in this PCR amplification contains 100ng of the cDNA encoding normal PR, a oligonucleotide depicted in SEQ ID:186 and a oligonucleotide depicted in SEQ ID:187, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). In this PCR amplification, there is repeated 25 times an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 55℃ for 3 minutes. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene). After recovering the amplified test cDNA from the low melting point agarose gel, 1μg of the amplified

test cDNA is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the amplified test cDNA. Subsequently, the resulting test cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends of the cDNA. After phenol treating the phosphorylated test cDNA, the phosphorylated test cDNA is ethanol precipitated to achieve a purified form of the phosphoylated test cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with BAP for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The restriction digested pRc/RSV is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated test cDNA are used in a ligation reaction with a T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid encoding normal PR. Such a plasmid is selected and is designated as pRc/RSV-hPR Kozak.

6.15. Example 15 Production of a plasmid encoding a human normal MR

A human liver cDNA library (CLONETECH, Quick clone cDNA#7113-1) is utilized to PCR amplify therefrom a cDNA encoding a normal MR (Genbank Accession No. M16801). The PCR mixture in this PCR amplification contains 10 ng of the human liver cDNA library, 10 pmol of an oligonucleotide depicted in SEQ ID:188, 10 pmol of an oligonucleotide depicted in SEQ ID:189, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP,

dGTP, dCTP). The oligonucleotides depicted in SEQ ID:188 and SEQ ID:189 are synthesized with a DNA synthesizer (Model 394, Applied Biosystems). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 60℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm with ethidium bromide staining, that the cDNA encoding the normal MR is PCR amplified. After recovering the amplified cDNA from the low melting point agarose gel, a sample of the recovered cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

Another PCR amplification is then conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA. The PCR mixture in this PCR amplification contains 100ng of the cDNA encoding the normal MR, a oligonucleotide depicted in SEQ ID:190 and a oligonucleotide depicted in SEQ ID:191, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). In this PCR amplification, there is repeated 25 times an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 60℃ for 3 minutes. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene). After recovering the amplified cDNA from the low melting point agarose gel, 1μg of the amplified cDNA is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the amplified cDNA. Subsequently, the resulting cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends of the cDNA. After phenol treating the phosphorylated cDNA, the phosphorylated cDNA is ethanol precipitated to achieve a purified form of the phosphoylated cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with BAP for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The

restriction digested pRc/RSV is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated cDNA are used in a ligation reaction with a T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E.coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid encoding normal MR. The plasmid is selected and is designated as pRc/RSV-hMR Kozak.

6.16. Example 16 Production of a stably transformed cell which stably contains in one of its chromosomes the MMTV reporter gene

The plasmid pMSG (Pharmacia) is restriction digested with restriction enzymes *Hind III* and *Sma I* to provide a DNA fragment encoding a partial sequence of the MMTV-LTR region, which has a size of 1463 bp. The 1463 bp DNA fragment is then treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the 1463bp DNA fragment.

The plasmid pGL3 (Promega), which encodes the firefly luciferase gene, is restriction digested with restriction enzymes *Bgl II* and *Hind III* and is then treated with BAP at 65℃ for 1 hour. The restriction digestion reaction mixture is then subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene) to confirm that there is a DNA fragment having a nucleotide sequence encoding the firefly luciferase. The DNA fragment having the nucleotide sequence encoding the firefly luciferase is then recovered from the low melting point agarose gel. Subsequently, 100 ng of the recovered DNA fragment have the nucleotide sequence encoding firefly

luciferase and 1 μg of the 1463 bp DNA fragment are used in a ligation reaction with T4 DNA ligase. The ligation reaction mixture is then used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then restriction digested with restriction enzymes *Kpn I* and *Cla I*. The restriction digestion reaction mixtures are subjected to agarose gel electrophoresis to confirm that there is a plasmid which contains 1 copy of the 1463bp DNA fragment operably upstream from the DNA fragment have the nucleotide sequence encoding firefly luciferase (hereinafter referred to as the MMTV reporter gene). Such a plasmid is selected and is designated as pGL3-MMTV.

The plasmid pUCSV-BSD (Funakoshi) is restriction digested with restriction enzyme *BamH I* to prepare a DNA encoding a blasticidin S deaminase gene expression cassette. Further, the plasmid pGL3-MMTV is restriction digested with restriction enzyme *BamH I* and is then treated with BAP at 65℃ for 1 hour. The resulting DNA encoding the blasticidin S deaminase gene expression cassette and the restriction digested pGL3-MMTV are mixed together to be used in a ligation reaction with T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells. The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems) to confirm there is a plasmid which has a structure in which the DNA encoding a blasticidin S Deaminase gene expression cassette has been inserted into the *Bam HI* restriction site in pGL3-MMTV. Such a plasmid is selected and is designated as pGL3-MMTV-BSD.

In order to produce stably transformed cells which stably contain in one of its chromosomes the MMTV reporter gene (hereinafter referred to as the stably

transformed MMTV cassette cell), the plasmid pGL3-MMTV-BSD is linearized and introduced into HeLa cells.

The plasmid pGL3-MMTV-BSD is restriction digested with restriction enzyme *Sal I* to linearize pGL3-MMTV-BSD.

Approximately $5x10^5$ HeLa cells were cultured as host cells for 1 day using dishes having a diameter of about 10 cm (Falcon) in DMEM medium (Nissui Pharmaceutical Co.) containing 10% FBS at 37°C under the presence of 5% CO_2.

The linearized pGL3-MMTV-BSD is then introduced to the cultured HeLa cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions under the lipofection method included 5 hours of treatment, 7 μg/dish of the linearized pGL3-MMTV-BSD and 21 μl/dish of lipofectamine.

After the lipofection treatment, the DMEM medium is exchanged with DMEM medium containing 10% FBS and the transformed HeLa cells are cultured for about 36 hours. Next, the transformed HeLa cells are removed and collected from the dish by trypsin treatment and are transferred into a container containing a medium to which blasticidin S is added to a concentration of 16 μg/ml. The transformed HeLa cells are cultured in such medium containing blasticidin S for 1 month while exchanging the medium every 3 or 4 days to a fresh batch of the medium containing blasticidin S.

The resulting clones, which are able to proliferate and produce a colony having a diameter of from 1 to several mm, are transferred as a whole to the wells of a 96-well ViewPlate (Berthold) to which medium is previously dispensed thereto. The colonies of the clones are further cultured. When the clones proliferated to such a degree that they covered 50% or more of the bottom surface of each of the wells (about 5 days after the transfer), the clones are removed and collected by trypsin treatment. The clones then are divided into 2 subcultures. One of the subcultures is transferred to a 96-well ViewPlate, which is designated as the master plate. The other subculture is transferred to a 96-well ViewPlate, which is designated as the assay plate. The master plate and the assay plate contain medium so that the clones can be cultured. The master pate is continuously cultured under similar conditions.

The medium is then removed from the wells of the assay plate and the clones attached to the well walls are washed twice with PBS(-). A 5-fold diluted lysis buffer PGC50 (Toyo Ink) is added, respectively, to the clones in the wells of the assay plate at 20 µl per well. The assay plate is left standing at room temperature for 30 minutes and is set on a luminometer LB96P (Berthold), which is equipped with an automatic substrate injector. Subsequently, 50 µl of the substrate solution PGL100 (Toyo Ink) is automatically dispensed to the lysed clones in the assay plate to measure the luciferase activity therein with the luminometer LB96P. A plurality of the clones, which exhibited a high luciferase activity are selected therefrom.

Samples of the selected clones are then cultured at 37℃ for 1 to 2 weeks in the presence of 5% CO_2 using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium.

The plasmid pRc/RSV-hAR Kozak is then introduced to the samples of the selected clones by a lipofection method using lipofectamine (Life Technologies) to provide a second round of clones. According with the manual provided with the lipofectamine, the conditions under the lipofection method included 5 hours of treatment, 7 µg/dish of the plasmids above and 21 µl/dish of lipofectamine. A DMSO solution containing dihydrotestosterone (DHT), which is the natural cognate ligand of a normal AR, is then added to the resulting second clones so that the concentration of DHT in the medium is 10nM. After culturing the second clones for 2 days, the luciferase activity is measured, similarly to the above, for each of the second clones. The clone in the master plate, which provided the second clone exhibiting the highest induction of luciferase activity, is selected as the stably transformed MMTV cassette cell.

In this regard, the stably transformed MMTV cassette cell can be used in reporter assays with AR, GR, PR, MR and the like.

6.17. Example 17 Reporter Assay of the human normal AR as a human test AR

6.17.1. Preparation of stably transformed MMTV cassette cell

Approximately $2x10^6$ stably transformed MMTV cassette cells provided in 6.16., are cultured at 37℃ for 1 day in the presence of 5% CO_2 using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium.

For transient expression, the plasmid pRc/RSV-hAR Kozak is introduced into a subculture of the stably transformed MMTV cassette cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions under the lipofection method include 5 hours of treatment, 7 μg/dish of the pRc/RSV-hAR Kozak and 21 μl/dish of lipofectamine. After culturing the resulting cell subculture at 37℃ for 16 hours in the presence of 5% CO_2, the charcoal dextran FBS/E-MEM medium therein is exchanged to fresh batches of the charcoal dextran FBS/E-MEM medium to further culture the cell subculture for 3 hours. The cell subculture is then collected and uniformly suspended in charcoal dextran FBS/E-MEM medium to provide a subculture thereof.

6.17.2. Measurement of the activity for transactivation of the MMTV reporter gene

First DMSO solutions are prepared to contain various concentrations of flutamide. The flutamide is used in the first DMSO solution as an agonist directed to the normal AR. Further, second DMSO solutions are prepared to contain 10nM of DHT and the various concentrations of the flutamide. The flutamide is used in the second DMSO solution as an antagonist directed to the normal AR.

The first and second DMSO solutions are then mixed, respectively, with the subcultures prepared in the above 6.17.1., in the 96-well ViewPlates such that the concentration of the first or second DMSO solution in each of the wells is about 0.1% (v/v). Further, as a standard, a sample of the cell subculture which is provided in 6.17.1., is mixed with a DMSO solution containing DHT, in the wells of a 96-ViewPlate.

The cells are then cultured for 40 hours at 37℃ in the presence of 5% CO_2. A 5-fold diluted lysis buffer PGC50 (Toyo Ink) is added, respectively, to the subcultures in the wells at 50 μl per well. The 96-well ViewPlates are periodically and gently shook while being incubated at room temperature for 30 minutes. Ten microliters (10μl) of the

lysed cells are then transferred, respectively, to white 96-well sample plates (Berthold) and are set on a luminometer LB96P (Berthold), which is equipped with an automatic substrate injector. Subsequently, 50 µl of the substrate solution PGL100 (Toyo Ink) is automatically dispensed, respectively, to each of the lysed cells in the white 96-well sample plates to instantaneously measure for 5 seconds the luciferase activity therein with the luminometer LB96P.

Further, the above reporter assay can use as the test AR, a mutant AR. In this regard, a plasmid encoding a mutant AR is used instead of pRc/RSV-hAR Kozak. To provide the plasmid encoding the mutant AR, a Kozak consensus sequence is added operably upstream from a polynucleotide encoding a mutant AR and the resulting polynucleotide is inserted into a restriction site of *Hind III* in the plasmid pRc/RSV (Invitrogen), as similarly described above.

6.18. Example 18 Reporter Assay of a human normal GR as the human test GR

6.18.1. Preparation of stably transformed MMTV cassette cell

Approximately $2x10^6$ stably transformed MMTV cassette cells provided in 6.16., are cultured at 37℃ for 1 day in the presence of 5% CO_2 using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium.

For transient expression, the plasmid pRc/RSV-hGR Kozak is introduced into a subculture of the stably transformed MMTV cassette cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions under the lipofection method include 5 hours of treatment, 7 µg/dish of the pRc/RSV-hAR Kozak and 21 µl/dish of lipofectamine. After culturing the resulting cell subculture at 37℃ for 16 hours in the presence of 5% CO_2, the charcoal dextran FBS/E-MEM medium therein is exchanged to fresh batches of the charcoal dextran FBS/E-MEM medium to further culture the cell subculture for 3 hours. The cell subculture is then collected and uniformly suspended in charcoal dextran FBS/E-MEM medium.

6.18.2. Measurement of the activity for transactivation of the MMTV reporter gene

First DMSO solutions are prepared to contain various concentrations of pregnanolone 16α carbonitrile (PCN). The PCN is used in the first DMSO solutions as an agonist with the normal GR. Further, the second DMSO solutions are prepared to contain 10nM of corticosterone and the various concentrations of PCN. The PCN is used in the second DMSO solutions as an antagonist with the normal GR.

The first and second DMSO solutions are then mixed, respectively, with the cell subcultures prepared in the above 6.18.1., in wells of the 96-well ViewPlates such that the concentration of the first or second DMSO solution in each of the wells is about 0.1% (v/v). Further, as a standard, a sample of the cell subculture is mixed with a DMSO solution containing corticosterone in the wells of a 96-well ViewPlate.

The cells are then cultured for 40 hours at 37℃ in the presence of 5% CO_2. A 5-fold diluted lysis buffer PGC50 (Toyo Ink) is added, respectively, to the subcultures in the wells at 50 μl per well. The 96-well ViewPlates are periodically and gently shook while being incubated at room temperature for 30 minutes. Ten microliters (10μl) of the lysed cells are then transferred, respectively, to white 96-well sample plates (Berthold) and are set on a luminometer LB96P (Berthold), which is equipped with an automatic substrate injector. Subsequently, 50 μl of the substrate solution PGL100 (Toyo Ink) is automatically dispensed, respectively, to each of the lysed cells in the white 96-well sample plates to instantaneously measure for 5 seconds the luciferase activity therein with the luminometer LB96P.

Further, the above reporter assay can use as the test GR, a mutant GR. In this regard, a plasmid encoding the mutant GR is used instead of pRc/RSV-hGR Kozak. To provide the plasmid encoding the mutant GR, a Kozak consensus sequence is added operably upstream from a polynucleotide encoding a mutant GR and the resulting polynucleotide is inserted into a restriction site of *Hind III* in the plasmid pRc/RSV (Invitrogen), as similarly described above.

6.19. Example 19 Reporter Assay of human normal PR as the human test

PR

6.19.1. Preparation of stably transformed MMTV cassette cell

Approximately $2x10^6$ stably transformed MMTV cells provided in 6.16., are cultured at 37℃ for 1 day in the presence of 5% CO_2 using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium.

For transient expression the plasmid pRc/RSV-hPR Kozak is introduced into a subculture of the stably transformed MMTV cassette cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions under the lipofection method include 5 hours of treatment, 7 μg/dish of the pRc/RSV-hAR Kozak and 21 μl/dish of lipofectamine. After culturing the resulting cell subculture at 37℃ for 16 hours in the presence of 5% CO_2, the charcoal dextran FBS/E-MEM medium therein is exchanged to fresh batches of the charcoal dextran FBS/E-MEM medium to further culture each of the cell subculture for 3 hours. The cell subculture is then collected and uniformly suspended in charcoal dextran FBS/E-MEM medium.

6.19.2. Measurement of the activity for transactivation of the MMTV reporter gene

First DMSO solutions are prepared to contain various concentrations of RU486. The RU486 is used in the first DMSO solutions as an agonist directed to the normal PR. Further, second DMSO solutions are prepared to contain 10nM of progesterone and the various concentrations of RU486. The RU486 is used in the second DMSO solutions as an antagonist directed to the normal PR.

The first and second DMSO solutions are mixed, respectively, with the cell subcultures prepared in the above 6.19.1., in the 96-well ViewPlates such that the concentration of the first or second DMSO solution in each of the wells is about 0.1% (v/v). Further, as a standard, a sample of the cell subculture is mixed with a DMSO solution containing progesterone.

The cells are then cultured for 40 hours at 37℃ in the presence of 5% CO_2. A 5-fold diluted lysis buffer PGC50 (Toyo Ink) is added, respectively, to the cells in the wells at 50 μl per well. The 96-well ViewPlates are periodically and gently shook while

being incubated at room temperature for 30 minutes. Ten microliters (10μl) of the lysed cells are then transferred, respectively, to white 96-well sample plates (Berthold) and are set on a luminometer LB96P (Berthold), which is equipped with an automatic substrate injector. Subsequently, 50 μl/well of the substrate solution PGL100 (Toyo Ink) is automatically dispensed, respectively, to each of the lysed cells in the white 96-well sample plates to instantaneously measure for 5 seconds the luciferase activity therein with the luminometer LB96P.

Further, the above reporter assay can use as the test PR, a mutant PR. In this regard, a plasmid encoding the mutant PR is used instead of pRc/RSV-hPR Kozak. To provide the plasmid encoding the mutant PR, a Kozak consensus sequence is added operably upstream from a polynucleotide encoding a mutant PR and the resulting polynucleotide is inserted into a restriction site of *Hind III* in the plasmid pRc/RSV (Invitrogen), as similarly described above.

6.20. Example 20 Production of a plasmid encoding human normal ERβ

A human prostate cDNA library (CLONETECH, Quick clone cDNA#7123-1) is utilized to PCR amplify therefrom a cDNA encoding a human normal ERβ (Genbank Accession No. AB006590). The PCR mixture in this PCR amplification contains 10 ng of the human liver cDNA library, 10 pmol of an oligonucleotide depicted in SEQ ID:192, 10 pmol of an oligonucleotide depicted in SEQ ID:193, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). The oligonucleotides depicted in SEQ ID:192 and SEQ ID:193 are synthesized with a DNA synthesizer (Model 394, Applied Biosystems). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm with ethidium bromide staining, that the cDNA encoding human normal ERβ is PCR amplified. After recovering the amplified cDNA from the low melting point agarose gel, a sample of the recovered

cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

Another PCR amplification is then conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA. The PCR mixture in this PCR amplification contains 100ng of the cDNA, 10pmol of an oligonucleotide depicted in SEQ ID:194 and 10pmol of an oligonucleotide depicted in SEQ ID:195, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). In this PCR amplification, there is repeated 25 times an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene). After recovering the amplified cDNA from the low melting point agarose gel, 1μg of the amplified cDNA is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the amplified cDNA. Subsequently, the resulting cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends of the cDNA. After phenol treating the phosphorylated cDNA, the phosphorylated cDNA is ethanol precipitated to achieve a purified form of the phosphoylated cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with BAP for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The restriction digested pRc/RSV is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated cDNA are used in a ligation reaction with a T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An

aliquot sample of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid encoding human normal ERβ. Such a plasmid is selected and is designated as pRc/RSV-hERβ Kozak.

6.21. Example 21 Reporter Assay of human normal ERβ as the human test ERβ

6.21.1. Preparation of stably transformed ERE cassette cell

Approximately $2x10^6$ stably transformed ERE cassette cells provided in 6.3., are cultured at 37℃ for 1 day in the presence of 5% CO_2 using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium.

For transient expression, the plasmid pRc/RSV-hERβ Kozak is introduced into a subculture of the stably transformed ERE cassette cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions under the lipofection method include 5 hours of treatment, 7 μg/dish of the pRc/RSV-hERβ Kozak and 21 μl/dish of lipofectamine. After culturing the resulting cell subculture at 37℃ for 16 hours in the presence of 5% CO_2, the charcoal dextran FBS/E-MEM medium therein is exchanged to fresh batches of the charcoal dextran FBS/E-MEM medium to further culture the cell subculture for 3 hours. The cell subculture is then collected and uniformly suspended in charcoal dextran FBS/E-MEM medium.

6.21.2. Measurement of the activity for transactivation of the ERE reporter gene

First DMSO solutions are prepared to contain various concentrations of 4-hydroxytamoxifen. The 4-hydroxytamoxifen is used in the first DMSO solutions as an agonist directed to the human normal ERβ. Further, second DMSO solutions are prepared to contain 10nM of E2 and the various concentrations of 4-hydroxytamoxifen. The 4-hydrohytamoxifen is used in the second DMSO solutions as an antagonist directed to the ERβ.

The first and second DMSO solutions are mixed, respectively, with the subcultures prepared in the above 6.21.1., in the 96-well ViewPlates such that the concentration of the first or second DMSO solution in each of the wells is about 0.1% (v/v). Further, as a standard, a sample of the cells is mixed with DMSO solutions containing E2 in the wells of a 96-well ViewPlate.

The cells are then cultured for 40 hours at 37℃ in the presence of 5% CO_2. A 5-fold diluted lysis buffer PGC50 (Toyo Ink) is added, respectively, to the cells in the wells at 50 µl per well. The 96-well ViewPlates are periodically and gently shook while being incubated at room temperature for 30 minutes. Ten microliters (10µl) of the lysed cells are then transferred, respectively, to white 96-well sample plates (Berthold) and are set on a luminometer LB96P (Berthold), which is equipped with an automatic substrate injector. Subsequently, 50 µl/well of the substrate solution PGL100 (Toyo Ink) is automatically dispensed, respectively, to each of the lysed cells in the white 96-well sample plates to instantaneously measure for 5 seconds the luciferase activity therein with the luminometer LB96P.

Further, the above reporter assay can use as the test ERβ, a mutant ERβ. In this regard, a plasmid encoding the mutant ERβ is used instead of pRc/RSV-hERβ Kozak. To provide the plasmid encoding the mutant ERβ, a Kozak consensus sequence is added operably upstream from a polynucleotide encoding a mutant ERβ and the resulting polynucleotide is inserted into a restriction site of *Hind III* in the plasmid pRc/RSV (Invitrogen), as similarly described above.

6.22. Example 22 Production of a plasmid encoding a human normal TRα

A human liver cDNA library (CLONETECH, Quick clone cDNA#7113-1) is utilized to PCR amplify therefrom a cDNA encoding a human normal TRα (Genbank Accession No. M24748). The PCR mixture in this PCR amplification contains 10 ng of the human liver cDNA library, 10 pmol of an oligonucleotide depicted in SEQ ID:196, 10 pmol of an oligonucleotide depicted in SEQ ID:197, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). The oligonucleotides depicted in SEQ ID:196 and SEQ ID:197 are

synthesized with a DNA synthesizer (Model 394, Applied Biosystems). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm with ethidium bromide staining, that the cDNA encoding human normal TRα is PCR amplified. After recovering the amplified cDNA from the low melting point agarose gel, a sample of the recovered cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

Another PCR amplification is then conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA. The PCR mixture in this PCR amplification contains 100ng of the cDNA encoding human normal TRα, 10pmol of an oligonucleotide depicted in SEQ ID:198 and 10pmol of an oligonucleotide depicted in SEQ ID:199, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). In this PCR amplification, there is repeated 25 times an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene). After recovering the amplified cDNA from the low melting point agarose gel, 1μg of the amplified cDNA is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the amplified cDNA. Subsequently, the resulting cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends of the cDNA. After phenol treating the phosphorylated cDNA, the phosphorylated cDNA is ethanol precipitated to achieve a purified form of the phosphorylated cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with BAP for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The

restriction digested pRc/RSV is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated cDNA are used in a ligation reaction with a T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid encoding human normal TRα. Such a plasmid is selected and is designated as pRc/RSV-hTRαKozak.

6.23. Example 23 Production of a plasmid encoding a human normal TRβ

A human liver cDNA library (CLONETECH, Quick clone cDNA#7113-1) is utilized to PCR amplify therefrom a cDNA encoding a human normal TRβ (Genbank Accession No. M26747). The PCR mixture in this PCR amplification contains 10 ng of the human liver cDNA library, 10 pmol of an oligonucleotide depicted in SEQ ID:200, 10 pmol of an oligonucleotide depicted in SEQ ID:201, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). The oligonucleotides depicted in SEQ ID:200 and SEQ ID:201 are synthesized with a DNA synthesizer (Model 394, Applied Biosystems). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm with ethidium bromide staining, that the cDNA encoding human normal TRβ is PCR amplified. After recovering the

amplified cDNA from the low melting point agarose gel, a sample of the recovered cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

Another PCR amplification is then conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA. The PCR mixture in this PCR amplification contains 100ng of the cDNA encoding normal TRα, 10pmol of an oligonucleotide depicted in SEQ ID:202 and 10pmol of an oligonucleotide depicted in SEQ ID:203, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). In this PCR amplification, there is repeated 25 times an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the amplified cDNA from the low melting point agarose gel, 1μg of the amplified cDNA is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the amplified cDNA. Subsequently, the resulting cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends of the cDNA. After phenol treating the phosphorylated cDNA, the phosphorylated cDNA is ethanol precipitated to achieve a purified form of the phosphoylated cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with BAP for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The restriction digested pRc/RSV is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated cDNA are used in a ligation reaction with a T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The

clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid encoding human normal TRβ. Such a plasmid is selected and is designated as pRc/RSV-hTRβ Kozak.

6.24. Example 24 Production of a plasmid containing an DR4 reporter gene

An oligonucleotide depicted in SEQ ID:204 and an oligonucleotide having a nucleotide sequence complementary thereto are synthesized with a DNA synthesizer. The oligonucleotide depicted in SEQ ID: 204 is synthesized to encode one of the strands of an DR4. The second oligonucleotide is synthesized to have a nucleotide sequence complementary to the first oligonucleotide. The two oligonucleotides are annealed together to produce a DNA encoding a DR4 sequence (hereinafter referred to as the DR4 DNA). A T4 polynucleotide kinase is allowed to react with the DR4 DNA to phosphorylate the ends thereof. The DR4 DNA is then ligated together with a T4 DNA ligase to provide a DR4x5 DNA having a 5 tandem repeat of the DR4 sequence. The ligation reaction mixture is then subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene), and the DR4x5 DNA is recovered from the gel .

The plasmid pGL3-TATA provided in 6.2., is restriction digested with restriction enzyme *Sma I* and is then treated with BAP at 65℃ for 1 hour. The restriction digested reaction mixture is then subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the DNA fragment having a nucleotide sequence encoding firefly luciferase from the low melting point agarose gel, 100 ng of the recovered DNA fragment and 1 μg of the DR4x5 DNA are used in a ligation reaction. The resulting ligation reaction mixture is then used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some

of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then restriction digested with restriction enzymes *Kpn I* and *Xho I*. The restriction digestion reaction mixtures are subjected to agarose gel electrophoresis to confirm that there is a plasmid having a structure in which the DR4x5 DNA is inserted into the restriction site of restriction enzyme *Sma I* in the pGL3-TATA. Such a plasmid is selected and is designated as pGL3-TATA-DR4x5.

The plasmid pGL3-TATA-DR4x5 is then restriction digested with restriction enzyme *Sal I*. After a Blunting Kit (Takara Shuzo) is used to blunt the ends of the restriction digested pGL3-TATA-DR4x5, the restriction digested pGL3-TATA-DR4x5 is treated with BAP at 65℃ for 1 hour. The Blunting Kit is also used to blunt the ends of the DNA fragment encoding the blasticidin S deaminase gene (*BamH I - BamH I* fragment) provided in 6.2.

The DNA fragment encoding a blasticidin S deaminase gene expression cassette and the restriction digested pGL3-TATA-DR4x5 are then mixed together for a ligation reaction with T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems) to confirm whether there is a plasmid which has a structure in which the DNA encoding a blasticidin S deaminase gene expression cassette is inserted into the restriction site of restriction enzyme *Sal I* in pGL3-TATA-DR4x5. Such a plasmid is selected and is designated as pGL3-TATA-DR4x5-BSD.

6.25. Example 25 Production of a stably transformed cell which stably contains in one of its chromosomes the DR4 reporter gene

In order to produce stably transformed cells which stably contains in one of its

chromosomes the DR4 reporter gene (hereinafter referred to as the stably transformed DR4 cassette cell, the plasmid pGL3-TATA-DR4x5-BSD was linearized and introduced into HeLa cells.

The plasmid pGL3-TATA-DR4x5-BSD is restriction digested with restriction enzyme *Not I* to linearize pGL3-TATA-DR4x5-BSD.

Approximately $5x10^5$ HeLa cells are cultured as host cells for 1 day using dishes having a diameter of about 10 cm (Falcon) in DMEM medium (Nissui Pharmaceutical Co.) containing 10% FBS at 37°C in the presence of 5% CO_2.

The linearized pGL3-TATA-DR4x5-BSD is then introduced to the cultured HeLa cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions under the lipofection method include 5 hours of treatment, 7 μg/dish of the linearized pGL3-TATA-DR4x5-BSD and 21 μl/dish of lipofectamine.

After the lipofection treatment, the DMEM medium is exchanged with DMEM medium containing 10% FBS and the transformed HeLa cells are cultured for about 36 hours. Next, the transformed HeLa cells are removed and collected from the dish by trypsin treatment and are transferred into a container containing a medium to which blasticidin S is added to a concentration of 16 μg/ml. The transformed HeLa cells are cultured in such medium containing blasticidin S for 1 month while exchanging the medium containing blasticidin S every 3 or 4 days to a fresh batch of the medium containing blasticidin S.

The resulting clones, which are able to proliferate and produce a colony having a diameter of from 1 to several mm, are transferred, respectively, as a whole to the wells of a 96-well ViewPlate (Berthold) to which medium is previously dispensed thereto. The clones are further cultured. When the clones proliferated to such a degree that clones therein covered 50% or more of the bottom surface of each of the wells (about 5 days after the transfer), the clones are removed and collected by trypsin treatment. Each of the clones then are divided into 2 subcultures. One of the subcultures is transferred to a 96-well ViewPlate, which is designated as the master plate. The other subculture was transferred to a 96-well ViewPlate, which is designated as the assay

plate. The master plate and the assay plate contain medium so that the clones can be cultured. The master pate is continuously cultured under similar conditions.

The medium in the wells of the assay plate is then removed therefrom and the clones attached to the well walls are washed twice with PBS(-). A 5-fold diluted lysis buffer PGC50 (Toyo Ink) is added, respectively, to the clones in the wells of the assay plate at 20 μl/ well. The assay plate is left standing at room temperature for 30 minutes and is set on a luminometer LB96P (Berthold), which is equipped with an automatic substrate injector. Subsequently, 50 μl of the substrate solution PGL100 (Toyo Ink) is automatically dispensed to the lysed clones in the assay plate to measure the luciferase activity therein with the luminometer LB96P. A plurality of the clones, which exhibited a luciferase activity are selected therefrom.

Samples of the selected clones are then cultured at 37℃ for 1 day in the presence of 5% CO_2 using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium.

The plasmid pRc/RSV-hTRαKozak is then introduced to the samples of the selected clones by a lipofection method using lipofectamine (Life Technologies) to provide a second round of clones. According with the manual provided with the lipofectamine, the conditions under the lipofection method included 5 hours of treatment, 7 μg/dish of pRc/RSV-hTRαKozak above and 21 μl/dish of lipofectamine. A DMSO solution containing triiodothyronine (T3), which is the natural cognate ligand of a human normal TRα, is then added to the resulting second clones so that the concentration of T3 in the medium is 10nM. After culturing the second clones for 2 days, the luciferase activity is measured, similarly to the above, for each of the second clones. The clone in the master plate, which provided the second clone exhibiting the highest induction of luciferase activity, is selected as the stably transformed DR4 cassette cell.

In this regard, the stably transformed DR4 cassette cell can be used in reporter assays with TRα, TRβ, CAR, LXR, PXR and the like.

6.26. Example 26 Production of a plasmid encoding human normal VDR

A human liver cDNA library (CLONETECH, Quick clone cDNA#7113-1) is utilized to PCR amplify therefrom a cDNA encoding a human normal VDR (Genbank Accession No. J03258). The PCR mixture in this PCR amplification contains 10 ng of the human liver cDNA library, 10 pmol of an oligonucleotide depicted in SEQ ID:205, 10 pmol of an oligonucleotide depicted in SEQ ID:206, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). The oligonucleotides depicted in SEQ ID:205 and SEQ ID:206 are synthesized with a DNA synthesizer (Model 394, Applied Biosystems). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene) to confirm with ethidium bromide staining, that the cDNA encoding human normal VDR is PCR amplified. After recovering the amplified cDNA from the low melting point agarose gel, a sample of the recovered cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

Another PCR amplification is then conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA encoding normal. The PCR mixture in this PCR amplification contains 100ng of the cDNA encoding normal, 10pmol of an oligonucleotide depicted in SEQ ID:207 and 10pmol of an oligonucleotide depicted in SEQ ID:208, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). In this PCR amplification, there is repeated 25 times an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the amplified cDNA from the low melting point agarose gel, 1μg of the amplified cDNA is treated with a DNA Blunting Kit

(Takara Shuzo) to blunt the ends of the amplified cDNA. Subsequently, the resulting cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends of the cDNA. After phenol treating the phosphorylated cDNA, the phosphorylated cDNA is ethanol precipitated to achieve a purified form of the phosphorylated cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with BAP for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The restriction digested pRc/RSV is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated cDNA is used in a ligation reaction with a T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid encoding human normal VDR. Such a plasmid is selected and is designated as pRc/RSV-hVDR Kozak.

6.27. Example 27 Production of a plasmid containing a DR3 reporter gene

An oligonucleotide depicted in SEQ ID:209 and an oligonucleotide having a nucleotide sequence complementary thereto are synthesized with a DNA synthesizer. The oligonucleotide depicted in SEQ ID: 209 is synthesized to encode one of the strands of a DR3. The second oligonucleotide is synthesized to have a nucleotide sequence complementary to the first oligonucleotide. The two oligonucleotides are annealed together to produce a DNA encoding a DR3 sequence (hereinafter referred to

as the DR3 DNA). A T4 polynucleotide kinase is allowed to react with the DR3 DNA to phosphorylate the ends thereof .The DR3 DNA is then ligated together with a T4 DNA ligase to provide a DR3x5 DNA having a 5 tandem repeat of the DR3 sequence. The ligation reaction mixture is then subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene), and the DR3x5 DNA is recovered from the gel .

The plasmid pGL3-TATA provided in 6.2., is restriction digested with restriction enzyme *Sma I* and is then treated with BAP at 65℃ for 1 hour. The restriction digested reaction mixture is then subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the DNA fragment having a nucleotide sequence encoding firefly luciferase from the low melting point agarose gel, 100 ng of the recovered DNA fragment and 1 μg of the DR3x5 DNA are used in a ligation reaction. The resulting ligation reaction mixture is then used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids is then restriction digested with restriction enzymes *Kpn I* and *Xho I*. The restriction digestion reaction mixtures are subjected to agarose gel electrophoresis to confirm that there is a plasmid having a structure in which the DR3x5 DNA is inserted into the restriction site of restriction enzyme *Sma I* in the pGL3-TATA. Such a plasmid is selected and is designated as pGL3-TATA-DR3x5.

The plasmid pGL3-TATA-DR3x5 is then restriction digested with restriction enzyme *Sal I*. After a Blunting Kit (Takara Shuzo) is used to blunt the ends of the restriction digested pGL3-TATA-DR3x5, the restriction digested pGL3-TATA-DR3x5 is treated with BAP at 65℃ for 1 hour. The Blunting Kit is also used to blunt the ends of the DNA fragment having the blasticidin S deaminase gene expression cassette (*BamH I - BamH I* fragment) provided in 6.2. The blunt ended pGL3-TATA-DR3x5 and the blunt ended DNA fragment having the blasticidin S deaminase gene expression cassette are used in a ligation reaction with T4 DNA ligase. The resulting ligation

reaction mixture is then used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. The isolated plasmid in which the DNA fragement having the blasticidn S deaminase gene expression cassette is inserted to the restriction site of restriction enzyme *Sal I* in pGL3-TATA-DR3x5 is selected and is designated as pGL3-TATA-DR3x5-BSD.

6.28. Example 28 Production of a stably transformed cassette cell which stably contains in one of its chromosomes the DR3 reporter gene

In order to produce stably transformed cells which stably contain in one of its chromosomes the DR3 reporter gene (hereinafter referred to as the stably transformed DR3 cassette cell, the plasmid pGL3-TATA-DR3x5-BSD was linearized and introduced into HeLa cells.

The plasmid pGL3-TATA-DR3x5-BSD is restriction digested with restriction enzyme *Not I* to linearize pGL3-TATA-DR3x5-BSD.

Approximately $5x10^5$ HeLa cells are cultured as host cells for 1 day using dishes having a diameter of about 10 cm (Falcon) in DMEM medium (Nissui Pharmaceutical Co.) containing 10% FBS at 37°C under the presence of 5% CO_2.

The linearized pGL3-TATA-DR3x5-BSD are then introduced to the cultured HeLa cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions under the lipofection method include 5 hours of treatment, 7 μg/dish of the linearized pGL3-TATA-DR3x5-BSD and 21 μl/dish of lipofectamine.

After the lipofection treatment, the DMEM medium is exchanged with DMEM medium containing 10% FBS and the transformed HeLa cells are cultured for about 36 hours. Next, the transformed HeLa cells are removed and collected from the dish by trypsin treatment and are transferred into a container containing a medium to which blasticidin S is added to a concentration of 16 μg/ml. The transformed cells are cultured in such medium containing blasticidin S for 1 month while exchanging the medium

containing blasticidin S every 3 or 4 days to a fresh batch of the DMEM medium containing blasticidin S.

The clones, which are able to proliferate and produce a colony having a diameter of from 1 to several mm, are transferred, respectively, as a whole to the wells of a 96-well ViewPlate (Berthold) to which medium is previously dispensed thereto. The clones are further cultured. When the clones proliferated to such a degree that eukaryotic clones therein covered 50% or more of the bottom surface of each of the wells (about 5 days after the transfer), the clones are removed and collected by trypsin treatment. The clones then are divided into 2 subcultures. One of the subcultures is transferred to a 96-well ViewPlate, which is designated as the master plate. The other subculture is transferred to a 96-well ViewPlate, which is designated as the assay plate. The master plate and the assay plate contain medium so that the clones can be cultured. The master pate is continuously cultured under similar conditions.

The medium in the wells of the assay plate is then removed therefrom and the clones attached to the well walls are washed twice with PBS(-). A 5-fold diluted lysis buffer PGC50 (Toyo Ink) is added, respectively, to the clones in the wells of the assay plate at 20 µl/well. The assay plate is left standing at room temperature for 30 minutes and is set on a luminometer LB96P (Berthold), which is equipped with an automatic substrate injector. Subsequently, 50 µl of the substrate solution PGL100 (Toyo Ink) is automatically dispensed to the lysed clones in the assay plate to measure the luciferase activity therein with the luminometer LB96P. A plurality of the clones, which exhibited a high luciferase activity are selected therefrom.

Samples of the selected clones are then cultured at 37℃ for 1 to 2 weeks in the presence of 5% CO_2 using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium.

The plasmid pRc/RSV-hVDR Kozak is then introduced to the samples of the selected clones by a lipofection method using lipofectamine (Life Technologies) to provide a second round of clones. According with the manual provided with the lipofectamine, the conditions under the lipofection method included 5 hours of treatment, 7 µg/dish of pRc/RSV-VDR Kozak above and 21 µl/dish of lipofectamine. A

DMSO solution containing 1,25-(OH) Vitamin D_3, which is the natural cognate ligand of a human normal VDR, is then added to the resulting second clones so that the concentration of 1,25-(OH) Vitamin D_3 in the medium is 10nM. After culturing the second clones for 2 days, the luciferase activity is measured, similarly to the above, for each of the second clones. The clone in the master plate, which provided the second clone exhibiting the highest induction of luciferase activity, is selected as the stably transformed DR3 cassette cell.

6.29. Example 29 Production of a plasmid encoding normal PPAR γ

A human liver cDNA library (CLONETECH, Quick clone cDNA#7113-1) is utilized to PCR amplify therefrom a cDNA encoding a human normal PPAR γ (Genbank Accession No. U79012). The PCR mixture in this PCR amplification contains 10 ng of the human liver cDNA library, 10 pmol of an oligonucleotide depicted in SEQ ID:210, 10 pmol of an oligonucleotide depicted in SEQ ID:211, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). The oligonucleotides depicted in SEQ ID:210 and SEQ ID:211 are synthesized with a DNA synthesizer (Model 394, Applied Biosystems). In this PCR amplification, there is repeated 35 times with a PCRsystem 9700 (Applied Biosystems), an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes.

The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L: Nippon Gene) to confirm with ethidium bromide staining, that the cDNA encoding human normal PPAR γ is PCR amplified. After recovering the amplified cDNA from the low melting point agarose gel, a sample of the recovered cDNA is prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The prepared sample of the cDNA is sequenced with an ABI autosequencer (Model 377, Applied Biosystems).

Another PCR amplification is then conducted to add a Kozak consensus sequence immediately upstream from the start codon (ATG) in the cDNA. The PCR mixture in this PCR amplification contains 100ng of the cDNA encoding normal PPAR

γ and Kozak consensus sequence, an oligonucleotide depicted in SEQ ID:212 and an oligonucleotide depicted in SEQ ID:211, LA-Taq Polymerase (Takara Shuzo), the buffer provided with the LA-Taq Polymerase and dNTPs (dATP, dTTP, dGTP, dCTP). In this PCR amplification, there is repeated 25 times an incubation cycle entailing an incubation at 95℃ for 1 minute followed by an incubation at 68℃ for 3 minutes. The resulting PCR mixture is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the amplified cDNA from the low melting point agarose gel, 1μg of the amplified cDNA is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends of the amplified cDNA. Subsequently, the resulting cDNA therefrom is allowed to react with a T4 polynucleotide kinase to phosphorylate the ends of the cDNA. After phenol treating the phosphorylated cDNA, the phosphorylated cDNA is ethanol precipitated to achieve a purified form of the phosphoylated cDNA.

The plasmid pRc/RSV (Invitrogen) is restriction digested with restriction enzyme *Hind III* and is then treated with BAP for 1 hour at 65℃. The restriction digested pRc/RSV is then purified by a phenol treatment and ethanol precipitation. The restriction digested pRc/RSV is treated with a DNA Blunting Kit (Takara Shuzo) to blunt the ends thereof and is subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the restriction digested pRc/RSV from the low melting point agarose gel, 100ng of the restriction digested pRc/RSV and all of the above purified form of the phosphorylated cDNA are used in a ligation reaction with a T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. Each of isolated plasmids is then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is a plasmid encoding human normal PPAR γ. Such a plasmid is selected and is designated as pRc/RSV-hPPAR γ Kozak.

6.30. Example 30 Production of a plasmid containing a DR1 reporter gene

An oligonucleotide depicted in SEQ ID:213 and an oligonucleotide having a nucleotide sequence complementary thereto are synthesized with a DNA synthesizer. The oligonucleotide depicted in SEQ ID: 213 is synthesized to encode one of the strands of a DR1 sequence. The second oligonucleotide is synthesized to have a nucleotide sequence complementary to the first oligonucleotide. The two oligonucleotides are annealed together to produce a DNA encoding a DR1 sequence (hereinafter referred to as the DR1 DNA). A T4 polynucleotide kinase is allowed to react with the DR1 DNA to phosphorylate the ends thereof. The DR1 DNA is then ligated together with a T4 DNA ligase to provide a DR1x5 DNA having a 5 tandem repeat of the DR1 sequence. The ligation reaction mixture is then subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene), and the DR1x5 DNA is recovered from the gel .

The plasmid pGL3-TATA provided in 6.2., is restriction digested with restriction enzyme *Sma I* and is then treated with BAP at 65℃ for 1 hour. The restriction digestion reaction mixture is then subjected to low melting point agarose gel electrophoresis (Agarose L, Nippon Gene). After recovering the DNA fragment having a nucleotide sequence encoding firefly luciferase from the low melting point agarose gel, 100 ng of the recovered DNA fragment and 1 μg of the DR1x5 DNA are used in a ligation reaction with T4 DNA ligase. The resulting ligation reaction mixture is then used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids is then restriction digested with restriction enzymes *Kpn I* and *Xho I*. The restriction digestion reaction mixtures are subjected to agarose gel electrophoresis to confirm that there is a plasmid in which the DR1x5 DNA is inserted into the restriction site of restriction enzyme *Sma I* in the pGL3-TATA. The plasmid is then sequenced with an ABI autosequencer (Model 377, Applied Biosystems), to confirm that there is provided

a plasmid having a 5 tandem repeat of the DR1 sequence. Such a plasmid is selected and is designated as pGL3-TATA-DR1x5.

The plasmid pGL3-TATA-DR1x5 is then restriction digested with restriction enzyme *Sal I*. After a Blunting Kit (Takara Shuzo) is used to blunt the ends of the restriction digested pGL3-TATA-DR1x5, the restriction digested pGL3-TATA-DR1x5 is treated with BAP at 65℃ for 1 hour. The Blunting Kit is also used to blunt the ends of the DNA fragment encoding the blasticidin S deaminase gene (*BamH I - BamH I* fragment derived from pUCSV-BSD (Funakoshi)) provided in 6.2.

The DNA fragment encoding a blasticidin S deaminase gene expression cassette and the restriction digested pGL3-TATA-DR1x5 are then mixed together for a ligation reaction with T4 DNA ligase. The ligation reaction mixture is used to transform E. coli competent DH5α cells (TOYOBO). The transformed E. coli cells are cultured in LB-amp. The clones thereof showing an ampicillin resistance are then recovered. Some of the clones are then used to isolate therefrom the plasmids derived from the ligation reaction. An aliquot sample of each of the isolated plasmids are then prepared with a Dye Terminator Sequence Kit FS (Applied Biosystems). The isolated plasmids are sequenced with an ABI autosequencer (Model 377, Applied Biosystems) to confirm whether the plasmid has a structure in which the DNA encoding a blasticidin S deaminase gene expression cassette has been inserted into the restriction site of restriction enzyme *Sal I* in pGL3-TATA-DR1x5. The plasmid is selected and is designated as pGL3-TATA-DR1x5-BSD.

6.31. Example 31 Production of a stably transformed cassette cell which stably contain in one of its chromosomes the DR1 reporter gene

In order to produce stably transformed cassette cells which stably contain in one of its chromosomes the DR1 reporter gene (hereinafter referred to as the stably transformed DR1 cassette cell), the plasmid pGL3-TATA-DR1x5-BSD is linearized and introduced into HeLa cells.

The plasmid pGL3-TATA-DR1x5-BSD is restriction digested with restriction enzyme *Not I* to linearize pGL3-TATA-DR1x5-BSD.

Approximately $5x10^5$ HeLa cells are cultured as host cells for 1 day using dishes having a diameter of about 10 cm (Falcon) in DMEM medium (Nissui Pharmaceutical Co.) containing 10% FBS at 37°C under the presence of 5% CO_2.

The linearized pGL3-TATA-DR1x5-BSD is then introduced to the cultured HeLa cells by a lipofection method using lipofectamine (Life Technologies). According with the manual provided with the lipofectamine, the conditions under the lipofection method include 5 hours of treatment, 7 μg/dish of the linearized pGL3-TATA-DR1x5-BSD and 21 μl/dish of lipofectamine.

After the lipofection treatment, the DMEM medium is exchanged with DMEM medium containing 10% FBS and the transformed HeLa cells are cultured for about 36 hours. Next, the transformed HeLa cells are removed and collected from the dish by trypsin treatment and are transferred into a container containing a medium to which blasticidin S is added to a concentration of 16 μg/ml. The transformed HeLa cells are cultured in such medium containing blasticidin S for 1 month while exchanging the medium containing blasticidin S every 3 or 4 days to a fresh batch of the medium containing blasticidin S.

The clones, which are able to proliferate and produce a colony having a diameter of from 1 to several mm, are transferred, respectively, as a whole to the wells of a 96-well ViewPlate (Berthold) to which medium is previously dispensed thereto. The clones are further cultured. When the clones proliferated to such a degree that clones therein covered 50% or more of the bottom surface of each of the wells (about 5 days after the transfer), the clones are removed and collected by trypsin treatment. The clones then are divided into 2 subcultures. One of the subcultures is transferred to a 96-well ViewPlate, which is designated as the master plate. The other subculture was transferred to a 96-well ViewPlate, which is designated as the assay plate. The master plate and the assay plate contain medium so that the clones can be cultured. The master pate is continuously cultured under similar conditions.

The medium in the wells of the assay plate is then removed therefrom and the clones attached to the well walls are washed twice with PBS(-). A 5-fold diluted lysis buffer PGC50 (Toyo Ink) is added, respectively, to the clones in the wells of the assay

plate at 20 μl/well. The assay plate is left standing at room temperature for 30 minutes and is set on a luminometer LB96P (Berthold), which is equipped with an automatic substrate injector. Subsequently, 50 μl of the substrate solution PGL100 (Toyo Ink) is automatically dispensed to the lysed clones in the assay plate to measure the luciferase activity therein with the luminometer LB96P. A plurality of clones, which exhibited a high luciferase activity are selected therefrom.

Samples of the selected clones are then cultured at 37℃ for 1 to 2 weeks in the presence of 5% CO_2 using dishes having a diameter of about 10 cm (Falcon) in charcoal dextran FBS/E-MEM medium.

The plasmid pRc/RSV-hPPAR γ Kozak is then introduced to the samples of the selected clones by a lipofection method using lipofectamine (Life Technologies) to provide a second round of clones. According with the manual provided with the lipofectamine, the conditions under the lipofection method included 5 hours of treatment, 7 μg/dish of pRc/RSV-hPPAR γ Kozak and 21 μl/dish of lipofectamine. A DMSO solution containing 15d prostagladin J2, which is the natural cognate.ligand of a human normal PPAR γ , is then added to the resulting second clones so that the concentration of 15d prostagladin J2 in the medium is 10nM. After culturing the second clones for 2 days, the luciferase activity is measured, similarly to the above, for each of the second clones. The clone in the master plate, which provided the second clone exhibiting the highest induction of luciferase activity, is selected as the stably transformed DR1 cassette cell.

In this regard, the stably transformed DR1 cassette cell can be used in reporter assays with PPAR, RAR, retinoin X receptor, HNF-4, TR-2, TR-4 and the like.

7. Sequence Free Text

SEQ ID:1	human normal ERα
SEQ ID:2	human mutant ERαK303R
SEQ ID:3	human mutant ERαS309F
SEQ ID:4	human mutant ERαG390D
SEQ ID:5	human mutant ERαM396V
SEQ ID:6	human mutant ERαG415V
SEQ ID:7	human mutant ERαG494V
SEQ ID:8	human mutant ERαK531E
SEQ ID:9	human mutant ERαS578P
SEQ ID:10	human mutant ERαG390D/S578P
SEQ ID:11	Designed oligonucleotide primer for PCR
SEQ ID:12	Designed oligonucleotide primer for PCR
SEQ ID:13	Designed oligonucleotide for mutagenesis
SEQ ID:14	Designed oligonucleotide for mutagenesis

SEQ ID:15 Designed oligonucleotide for mutagenesis

SEQ ID:16 Designed oligonucleotide for mutagenesis

SEQ ID:17 Designed oligonucleotide for mutagenesis

SEQ ID:18 Designed oligonucleotide for mutagenesis

SEQ ID:19 Designed oligonucleotide for mutagenesis

SEQ ID:20 Designed oligonucleotide for mutagenesis

SEQ ID:21 Designed oligonucleotide for mutagenesis

SEQ ID:22 Designed oligonucleotide for mutagenesis

SEQ ID:23 Designed oligonucleotide for mutagenesis

SEQ ID:24 Designed oligonucleotide for mutagenesis

SEQ ID:25 Designed oligonucleotide for mutagenesis

SEQ ID:26 Designed oligonucleotide for mutagenesis

SEQ ID:27 Designed oligonucleotide for mutagenesis

SEQ ID:28 Designed oligonucleotide for mutagenesis

SEQ ID:29 Designed oligonucleotide primer for PCR

SEQ ID:30 Designed oligonucleotide primer for PCR

SEQ ID:31 Designed oligonucleotide primer for PCR

SEQ ID:32 Designed oligonucleotide primer for PCR

SEQ ID:33 Designed oligonucleotide primer for PCR

SEQ ID:34 Designed oligonucleotide primer for PCR

SEQ ID:35 Designed oligonucleotide primer for PCR

SEQ ID:36 Designed oligonucleotide primer for PCR

SEQ ID:37 Designed oligonucleotide primer for PCR

SEQ ID:38 Designed oligonucleotide primer for PCR

SEQ ID:39 Designed oligonucleotide primer for PCR

SEQ ID:40 Designed oligonucleotide primer for PCR

SEQ ID:41 Designed oligonucleotide primer for PCR

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SEQ ID:57 Designed oligonucleotide primer for PCR

SEQ ID:58 Designed oligonucleotide primer for PCR

SEQ ID:59 Designed oligonucleotide primer for PCR

SEQ ID:60 Designed oligonucleotide primer for PCR

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SEQ ID:66 Designed oligonucleotide primer for PCR

SEQ ID:67 Designed oligonucleotide primer for PCR

SEQ ID:68 Designed oligonucleotide primer for PCR

SEQ ID:69 Designed oligonucleotide primer for PCR

SEQ ID:70 Designed oligonucleotide primer for PCR

SEQ ID:71 Designed oligonucleotide primer for PCR

SEQ ID:72 Designed oligonucleotide primer for PCR

SEQ ID:73 Designed oligonucleotide primer for PCR

SEQ ID:74 Designed oligonucleotide primer for PCR

SEQ ID:75 Designed oligonucleotide primer for PCR

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SEQ ID:100 Designed oligonucleotide primer for PCR

SEQ ID:101 Designed oligonucleotide primer for PCR

SEQ ID:102 Designed oligonucleotide primer for PCR

SEQ ID:103 Designed oligonucleotide primer for PCR

SEQ ID:104 Designed oligonucleotide primer for PCR

SEQ ID:105 Designed oligonucleotide primer for PCR

SEQ ID:106 Designed oligonucleotide primer for PCR

SEQ ID:107 Designed oligonucleotide primer for PCR

SEQ ID:108 Designed oligonucleotide primer for PCR

SEQ ID:109 Designed oligonucleotide primer for PCR

SEQ ID:110 Designed oligonucleotide primer for PCR

SEQ ID:111 Designed oligonucleotide probe for Southern hybridization

SEQ ID:112 Designed oligonucleotide probe for Southern hybridization

SEQ ID:113 Designed oligonucleotide probe for Southern hybridization

SEQ ID:114 Designed oligonucleotide probe for Southern hybridization

SEQ ID:115 Designed oligonucleotide probe for Southern hybridization

SEQ ID:116 Designed oligonucleotide probe for Southern hybridization

SEQ ID:117 Designed oligonucleotide probe for Southern hybridization

SEQ ID:118 Designed oligonucleotide probe for Southern hybridization

SEQ ID:119 Designed oligonucleotide probe for Southern hybridization

SEQ ID:120 Designed oligonucleotide probe for Southern hybridization

SEQ ID:121 Designed oligonucleotide probe for Southern hybridization

SEQ ID:122 Designed oligonucleotide probe for Southern hybridization

SEQ ID:123 Designed oligonucleotide probe for Southern hybridization

SEQ ID:124 Designed oligonucleotide probe for Southern hybridization

SEQ ID:125 Designed oligonucleotide probe for Southern hybridization

SEQ ID:126 Designed oligonucleotide probe for Southern hybridization

SEQ ID:127 Designed oligonucleotide probe for Southern hybridization

SEQ ID:128 Designed oligonucleotide probe for Southern hybridization

SEQ ID:129 Designed oligonucleotide probe for Southern hybridization

SEQ ID:130 Designed oligonucleotide probe for Southern hybridization

SEQ ID:131 Designed oligonucleotide probe for Southern hybridization

SEQ ID:132 Designed oligonucleotide probe for Southern hybridization

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SEQ ID:145 Designed oligonucleotide probe for Southern hybridization

SEQ ID:146 Designed oligonucleotide probe for Southern hybridization

SEQ ID:147 Designed oligonucleotide probe for Southern hybridization

SEQ ID:148 Designed oligonucleotide probe for Southern hybridization

SEQ ID:149 Designed oligonucleotide probe for Southern hybridization

SEQ ID:150 Designed oligonucleotide probe for Southern hybridization

SEQ ID:151 Designed oligonucleotide primer for PCR

SEQ ID:152 Designed oligonucleotide for mutagenesis

SEQ ID:153 Designed oligonucleotide for mutagenesis

SEQ ID:154 Designed oligonucleotide for mutagenesis

SEQ ID:155 Designed oligonucleotide for mutagenesis

SEQ ID:156 Designed oligonucleotide for mutagenesis

SEQ ID:157 Designed oligonucleotide for mutagenesis

SEQ ID:158 Designed oligonucleotide primer for PCR

SEQ ID:159 Designed oligonucleotide primer for PCR

SEQ ID:160 Designed oligonucleotide primer for PCR

SEQ ID:161 Designed oligonucleotide for synthesis

SEQ ID:162 Designed oligonucleotide for synthesis

SEQ ID:163 Designed oligonucleotide for synthesis

SEQ ID:164 Designed oligonucleotide primer for PCR

SEQ ID:165 Designed oligonucleotide primer for PCR

SEQ ID:166 Designed oligonucleotide primer for PCR

SEQ ID:167 Designed oligonucleotide primer for PCR

SEQ ID:168 Designed oligonucleotide primer for PCR

SEQ ID:169 Designed oligonucleotide primer for PCR

SEQ ID:170 Designed oligonucleotide primer for PCR

SEQ ID:171 Designed oligonucleotide primer for PCR

SEQ ID:172 Designed oligonucleotide primer for PCR

SEQ ID:173 Designed oligonucleotide primer for PCR

SEQ ID:174 Designed oligonucleotide primer for PCR

SEQ ID:175 Designed oligonucleotide primer for PCR

SEQ ID:176 Designed oligonucleotide primer for PCR

SEQ ID:177 Designed oligonucleotide primer for PCR

SEQ ID:178 Designed oligonucleotide primer for PCR

SEQ ID:179 Designed oligonucleotide primer for PCR

SEQ ID:180 Designed oligonucleotide primer for PCR

SEQ ID:181 Designed oligonucleotide primer for PCR

SEQ ID:182 Designed oligonucleotide primer for PCR

SEQ ID:183 Designed oligonucleotide primer for PCR

SEQ ID:184 Designed oligonucleotide primer for PCR

SEQ ID:185 Designed oligonucleotide primer for PCR

SEQ ID:186 Designed oligonucleotide primer for PCR

SEQ ID:187 Designed oligonucleotide primer for PCR

SEQ ID:188 Designed oligonucleotide primer for PCR

SEQ ID:189 Designed oligonucleotide primer for PCR

SEQ ID:190 Designed oligonucleotide primer for PCR

SEQ ID:191 Designed oligonucleotide primer for PCR

SEQ ID:192 Designed oligonucleotide primer for PCR

SEQ ID:193 Designed oligonucleotide primer for PCR

SEQ ID:194 Designed oligonucleotide primer for PCR

SEQ ID:195 Designed oligonucleotide primer for PCR

SEQ ID:196 Designed oligonucleotide primer for PCR

SEQ ID:197 Designed oligonucleotide primer for PCR

SEQ ID:198 Designed oligonucleotide primer for PCR

SEQ ID:199 Designed oligonucleotide primer for PCR

SEQ ID:200 Designed oligonucleotide primer for PCR

SEQ ID:201 Designed oligonucleotide primer for PCR

SEQ ID:202 Designed oligonucleotide primer for PCR

SEQ ID:203 Designed oligonucleotide primer for PCR

SEQ ID:204 Designed oligonucleotide for synthesis

SEQ ID:205 Designed oligonucleotide primer for PCR

SEQ ID:206 Designed oligonucleotide primer for PCR

SEQ ID:207 Designed oligonucleotide primer for PCR

SEQ ID:208 Designed oligonucleotide primer for PCR

SEQ ID:209 Designed oligonucleotide for synthesis

SEQ ID:210 Designed oligonucleotide primer for PCR

SEQ ID:211 Designed oligonucleotide primer for PCR

SEQ ID:212 Designed oligonucleotide primer for PCR

SEQ ID:213 designed oligonucleotide for synthesis

CLAIMS:

1. An artificial cell comprising:

(i) a chromosome which comprises a reporter gene, wherein the reporter gene comprises an ERE, a TATA sequence and a reporter sequence naturally foreign to the ERE; and

(ii) one or more factors selected from the following (ii-a) and (ii-b):

(ii-a) a mutant ERα which has an activity for transactivation of the reporter gene, wherein

in the presence of a partial anti-estrogen and E2 the activity is higher than that of a normal ERα in the presence of the partial anti-estrogen and E2 or

in the presence of a partial anti-estrogen the activity is higher than that of a normal ERα in the presence of the partial anti-estrogen; and

(ii-b) a gene which encodes the mutant ERα.

2. The artificial cell according to claim 1, wherein the gene of (ii-b) is comprised by the chromosome of (i).

3. The artificial cell according to claim 1, wherein the gene of (ii-b) is comprised by a vector.

4. The artificial cell according to claim 1, wherein the partial anti-estrogen is tamoxifen, raloxifene or 4-hydroxytamoxifen.

5. The artificial cell according to claim 1, wherein the activity is also an activity for transactivation of the reporter gene which is inhibited in the presence of a pure anti-estrogen.

6. The artificial cell according to claim 1, wherein the mutant ERα has one or more substituted amino acids which confer the activity.

7. The artificial cell according to claim 1, wherein the mutant ERα has one or more substituted amino acids which confer the activity at one or more relative positions from 303 to 578, wherein the relative positions are based on a homology alignment to an amino acid sequence shown in SEQ ID:1.

8. The artificial cell according to claim 1, wherein the mutant ERα has one or more substituted amino acids which confers the activity at one or more relative positions selected from 303, 309, 390, 396, 415, 494, 531 and 578, wherein the relative positions are based on a homology alignment to an amino acid sequence shown in SEQ ID:1.

9. The artificial cell according to claim 1, wherein the normal ERα is an ERα

having an amino acid sequence shown in SEQ ID:1.

10. An artificial cell comprising:

a chromosome which comprises a reporter gene, wherein the reporter gene comprises an ERE, a TATA sequence and a reporter sequence naturally foreign to the ERE; and

a mutant ERα which activates transcription of a gene downstream from an ERE while exposed to an anti-estrogen which is not antagonistic to an AF1 region of a normal ERα and is antagonistic to an AF2 region of a normal ERα.

11. The artificial cell according to claim 10, wherein the mutant ERα is one which also activates transcription of the gene downstream from an ERE while bound to E2, wherein the activation is not inhibited by the anti-estrogen which is not antagonistic to an AF1 region of a normal ERα and is antagonistic to an AF2 region of a normal ERα.

12. An isolated mutant ERα having:

an activity for transactivation of a reporter gene, the reporter gene comprising an ERE, a TATA sequence and a reporter sequence naturally foreign to the ERE, wherein

in the presence of a partial anti-estrogen and E2 the activity is higher than that of a normal ERα in the presence of the partial anti-estrogen and E2, or

in the presence of a partial anti-estrogen the activity is higher than that of a normal ERα in the presence of the partial anti-estrogen; and

an amino acid sequence of an ERα comprising one or more substituted amino acids at one or more relative positions selected from 303, 309, 390, 396, 494 and 578, or two or more substituted amino acids at two or more relative positions selected from 303, 309, 390, 396, 415, 494, 531 and 578, wherein the relative positions are based on a homology alignment to an amino acid sequence shown in SEQ ID:1.

13. The isolated mutant ERα according to claim 12, wherein the substituted amino acid is an arginine at relative position 303, a phenylalanine at relative position 309, an asparaginic acid at relative position 390, a valine at relative position 396, a valine at relative position 494 or a proline at a relative position 578, wherein the relative positions are based on a homology alignment to an amino acid sequence shown in SEQ ID:1.

14. The isolated mutant ERα according to claim 12, which is derived from a normal ERα comprising a lysine at relative position 303, a serine at relative position 309, a glycine at relative position 390, a methionine at relative position 396, a glycine at relative position 494 and a serine at relative position 578, wherein the relative positions are based on a homology alignment to an amino acid sequence shown in SEQ ID:1.

15. An isolated mutant ERα having an amino acid sequence shown in SEQ ID:2.

16. An isolated mutant ERα having an amino acid sequence shown in SEQ ID:3.

17. An isolated mutant ERα having an amino acid sequence shown in SEQ ID:4.

18. An isolated mutant ERα having an amino acid sequence shown in SEQ ID:5.

19. An isolated mutant ERα having an amino acid sequence shown in SEQ ID:7.

20. An isolated mutant ERα having an amino acid sequence shown in SEQ ID:9.

21. An isolated mutant ERα having an amino acid sequence shown in SEQ ID:10.

22. An isolated polynucleotide encoding the mutant ERα of claim 12.

23. An isolated polynucleotide encoding the mutant ERα of claim 15.

24. An isolated polynucleotide encoding the mutant ERα of claim 16.

25. An isolated polynucleotide encoding the mutant ERα of claim 17.

26. An isolated polynucleotide encoding the mutant ERα of claim 18.

27. An isolated polynucleotide encoding the mutant ERα of claim 19.

28. An isolated polynucleotide encoding the mutant ERα of claim 20.

29. An isolated polynucleotide encoding the mutant ERα of claim 21.

30. A vector comprising the polynucleotide of claim 22.

31. A virus comprising the vector of claim 30.

32. A method for quantitatively analyzing an activity for transactivation of a reporter gene by a test ERα, the method comprising:

exposing an artificial cell with a ligand, the artificial cell comprising the test ERα and a chromosome which comprises the reporter gene, wherein the reporter gene comprises an ERE, a TATA sequence and a reporter sequence naturally foreign to the ERE; and

measuring a transactivation amount of the reporter gene by the test ERα.

33. The method according to claim 32, wherein the ligand is a partial anti-

estrogen.

34. The method according to claim 32, wherein the ligand is tamoxifen, raloxifene or 4-hydroxy-tamoxifen.

35. The method according to claim 32, wherein the ligand is an anti-estrogen which is not antagonistic to an AF1 region and is antagonistic to an AF2 region.

36. A method for screening a test ligand dependent transcriptional factor, the method comprising:

exposing an artificial cell with a ligand, the artificial cell comprising a test ligand dependent transcriptional factor and a chromosome which comprises the reporter gene, wherein the reporter gene comprises a receptor responsive sequence cognate with the test ligand dependent transcriptional factor, a TATA sequence and a reporter sequence naturally foreign to the receptor responsive sequence;

measuring a transactivation amount of the reporter gene by the test ligand dependent transcriptional factor;

comparing the transactivation amount of the reporter gene by the test ligand dependent transcriptional factor to a transactivation amount of the reporter gene by a standard; and

selecting the test ligand dependent transcriptional factor wherein the transactivation amount of the reporter gene by the test transactivational amount is different than the transactivation amount of the reporter gene by the standard.

37. The method according to claim 36, wherein the test ligand dependent transcriptional factor is a mutant ligand dependent transcriptional factor and the standard is a normal test ligand dependent transcriptional factor.

38. The method according to claim 36, wherein the test ligand dependent transcriptional factor is a test ERα, a test ER β, a test AR, a test GR, a test PR, a test MR, a test receptor naturally having a lipophilic vitamin as a ligand, a test PPAR or a test TR.

39. A method for screening a test ERα, the method comprising:

exposing an artificial cell with a ligand, the artificial cell comprising the test ERα and a chromosome which comprises the reporter gene, wherein the reporter gene comprises an ERE, a TATA sequence and a reporter sequence naturally foreign the ERE;

measuring a transactivation amount of the reporter gene by the test ERα;

comparing the transactivation amount of the reporter gene by the test ERα to a transactivational amount of the reporter gene by a standard; and

selecting the test ERα wherein the transactivation amount of the reporter gene by the test ERα is different than the transactivation amount of the reporter gene by the standard.

40. The method according to claim 39, wherein the standard is a normal ERα, a normal ERα having an amino acid sequence shown in SEQ ID:1 or an ERα which phenotype is known.

41. The method according to claim 39, wherein the ligand is a partial anti-estrogen.

42. The method according to claim 39, wherein the ligand is tamoxifen, raloxifene or 4-hydroxy-tamoxifen.

43. A method for evaluating an activity for transactivation of a reporter gene by a test ERα, the method comprising:

exposing an artificial cell with a ligand, the artificial cell comprising the test ERα and a chromosome which comprises the reporter gene, wherein the reporter gene comprises an ERE, a TATA sequence and a reporter sequence naturally foreign the ERE;

measuring a transactivation amount of the reporter gene by the test ERα; and

comparing the transactivation amount of the reporter gene by the test ERα to a transactivational amount of the reporter gene by a standard.

44. The method according to claim 43, wherein the standard is a normal ERα, a normal ERα having an amino acid sequence shown in SEQ ID:1 or an ERα which

phenotype is known.

45. The method according to claim 43, wherein the ligand is a partial anti-estrogen.

46. The method according to claim 43, wherein the ligand is tamoxifen, raloxifene or 4-hydroxy-tamoxifen.

47. A method for screening a compound useful for treating a disorder of a mutant ERα, the method comprising:

exposing the artificial cell of claim 1 with a test compound;

measuring a transactivation amount of the reporter gene of the artificial cell.

48. A pharmaceutical agent useful for treating a disorder of a mutant ERα, the agent comprising as an active ingredient a compound screened by the method of claim 46.

49. Use of the mutant ERα of claim 12 for a receptor binding assay.

50. A method for diagnosing a genotype of a polynucleotide encoding a test ERα, the method comprising:

searching in a nucleotide sequence of the polynucleotide encoding the test ERα for one or more variant codons which encode one or more substituted amino acids

in the test ERα, wherein the one or more substituted amino acids confer an activity for transactivation of a reporter gene,

in which in the presence of a partial anti-estrogen and E2 the activity is higher than that of a normal ERα in the presence of the partial anti-estrogen and E2 or which in the presence of the partial anti-estrogen the activity is higher than that of a normal ERα in the presence of the partial anti-estrogen, and

in which a chromosome comprises the reporter gene, the reporter gene comprising an ERE, a TATA sequence and a reporter sequence naturally foreign the ERE; and

determining the mutation in the nucleotide sequence of the one or more variant codons by comparing the nucleotide sequence of the one or more variant codons to a nucleotide sequence of one or more corresponding codons in a nucleotide sequence encoding a standard.

51. The method according to claim 50, wherein the nucleotide sequence of the polynucleotide encoding the test ERα is searched at one or more variant codons which encode an amino acid at one or more relative positions selected from 303, 309, 390, 396, 494 and 578, or an amino acid at two or more relative positions selected from 303, 309, 390, 396, 415, 494, 531 and 578, wherein the relative positions are based on a homology alignment to an amino acid sequence shown in SEQ ID:1.

52. The method according to claim 50, wherein the standard is a normal ERα, a

normal ERα having an amino acid sequence shown in SEQ ID:1 or an ERα which phenotype is known.

53. A method for diagnosing a phenotype of a test ERα, the method comprising:

searching in an amino acid sequence of the test ERα for one or more substituted amino acids in the test ERα, wherein the one or more substituted amino acids confer an activity for transactivation of a reporter gene,

in which in the presence of a partial anti-estrogen and E2 the activity is higher than that of a normal ERα in the presence of the partial anti-estrogen and E2 or which in the presence of a partial anti-estrogen the activity is higher than that of a normal ERα in the presence of the partial anti-estrogen, and

in which a chromosome comprises the reporter gene, the reporter gene comprising an ERE, a TATA sequence and a reporter sequence naturally foreign to the ERE; and

determining the mutation in the amino acid sequence of the test ERα by comparing the amino acid sequence of the test ERα to an amino acid sequence of a standard.

54. The method according to claim 53, wherein the an amino acid sequence of the test ERα is searched at one or more relative positions selected from 303, 309, 390, 396, 494 and 578, or at two or more relative positions selected from 303, 309, 390, 396, 415, 494, 531 and 578, wherein the relative positions are based on a homology alignment to

an amino acid sequence shown in SEQ ID:1.

55. The method according to claim 53, wherein the standard is a normal ERα, a normal ERα having an amino acid sequence shown in SEQ ID:1 or an ERα which phenotype is known.

DRAWINGS

Figure 1

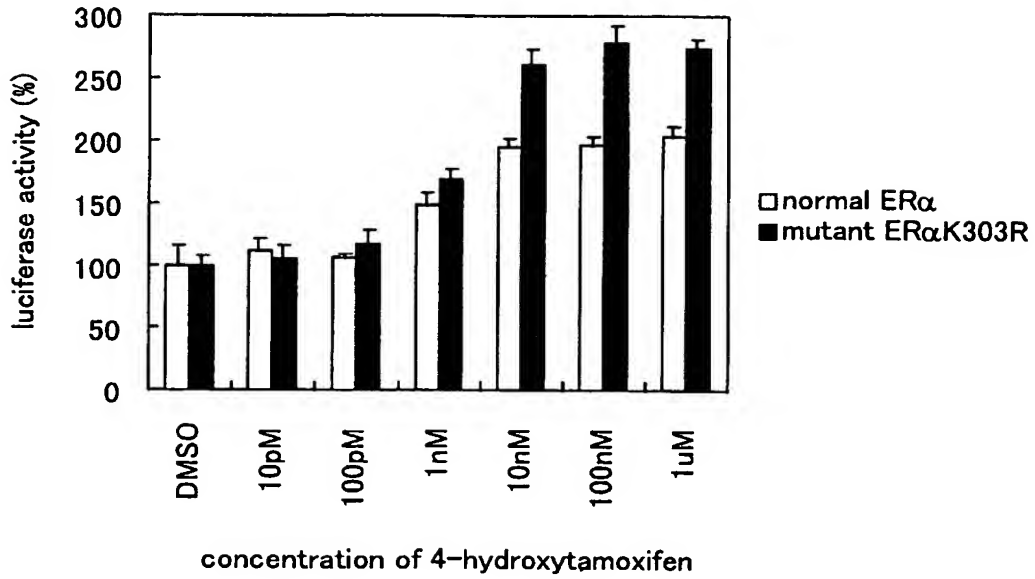
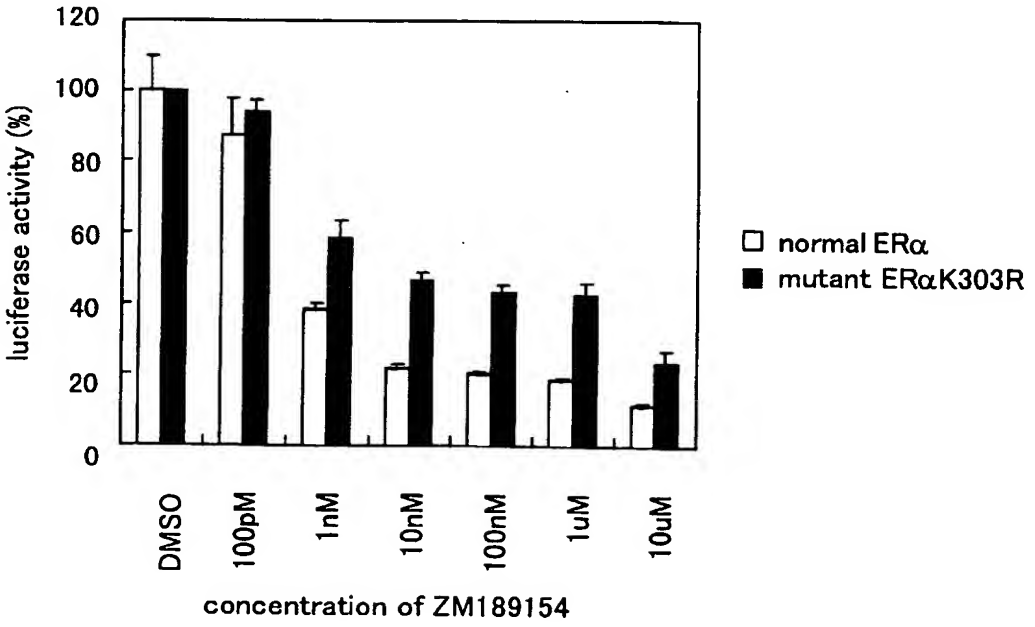


Figure 2



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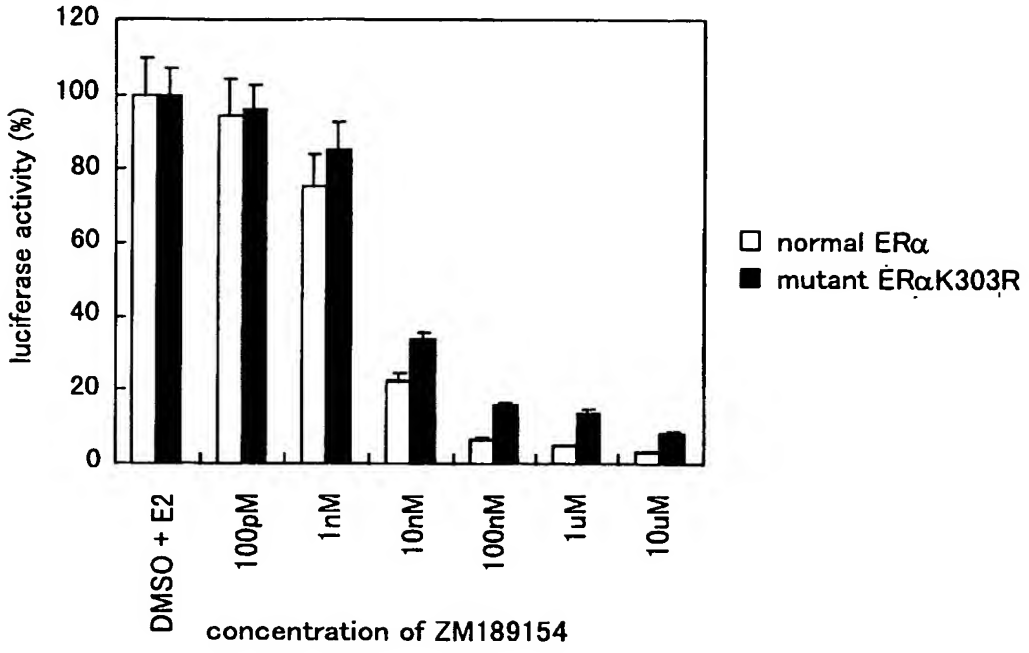

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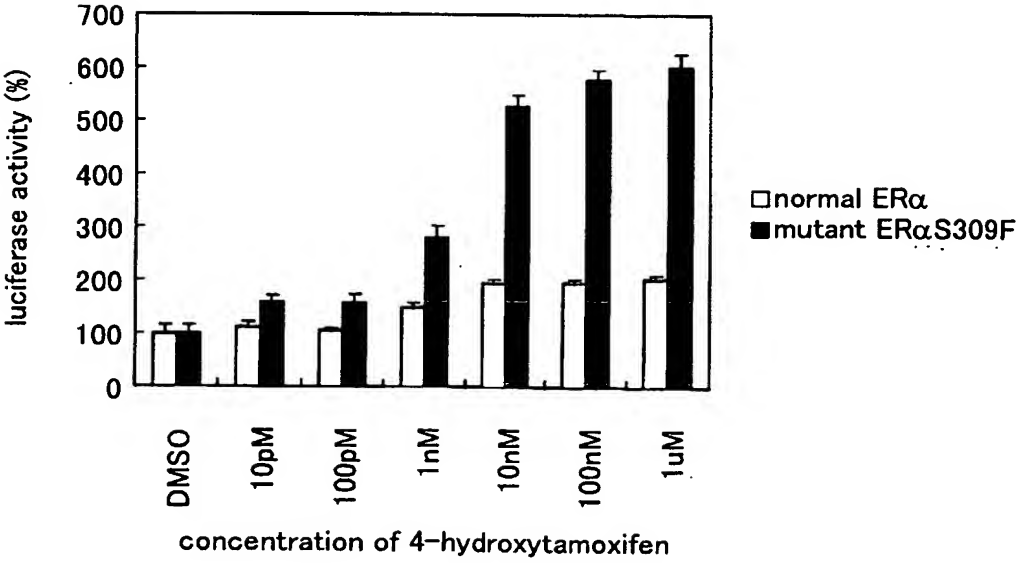

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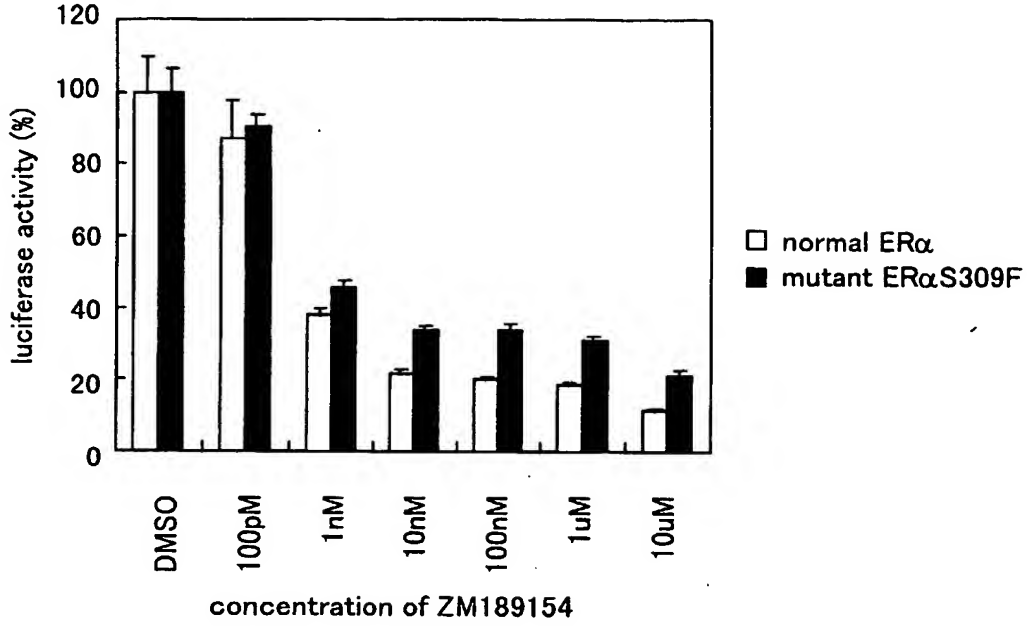

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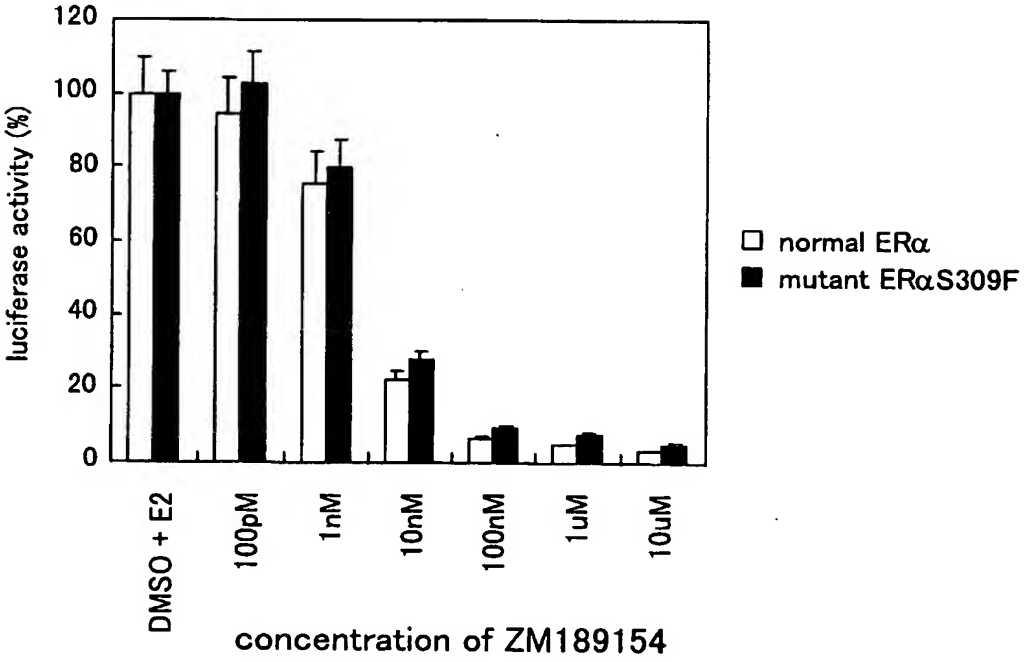

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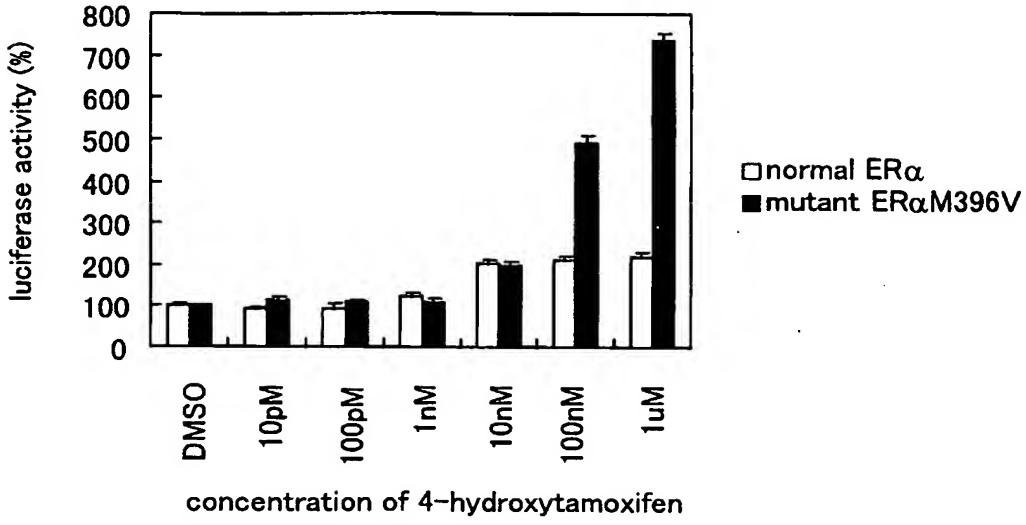

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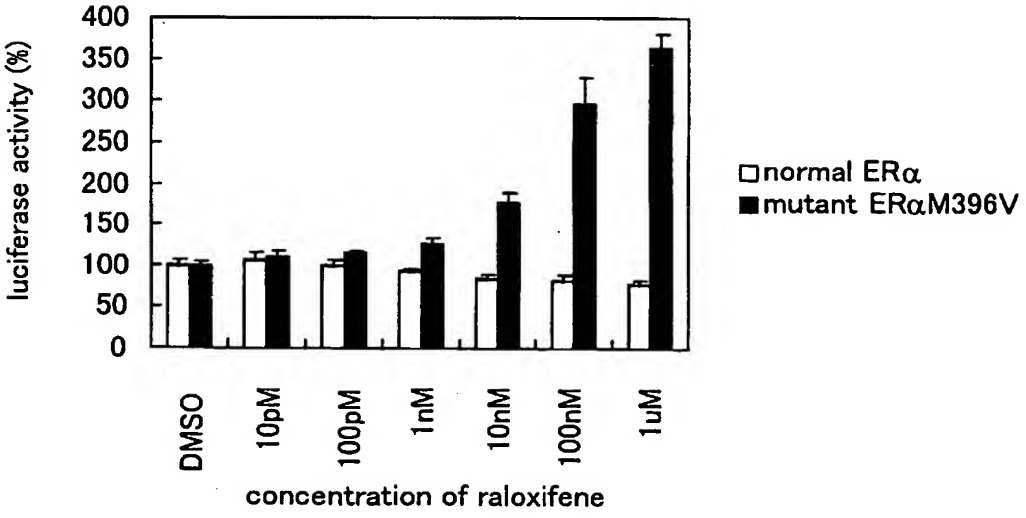

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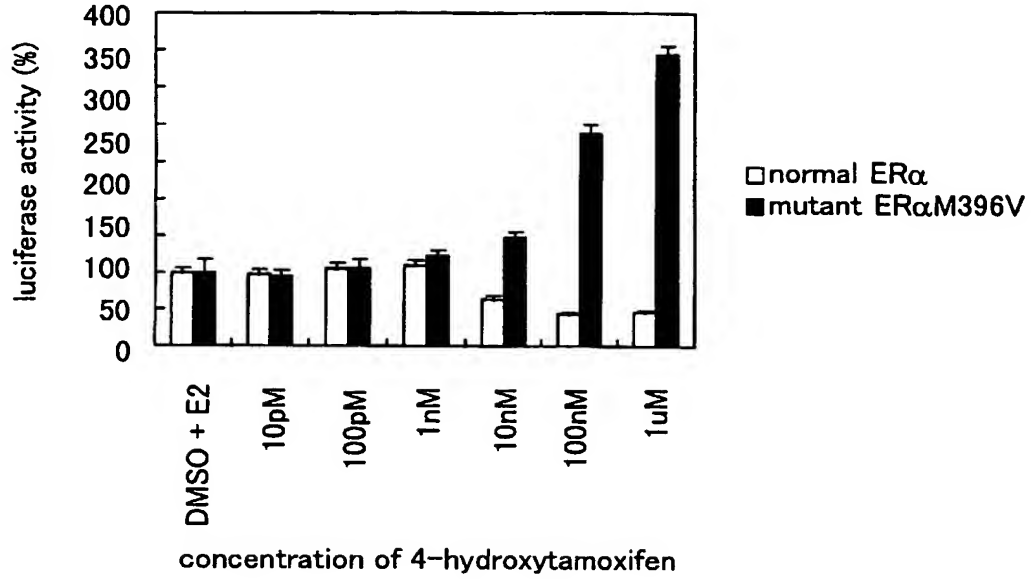

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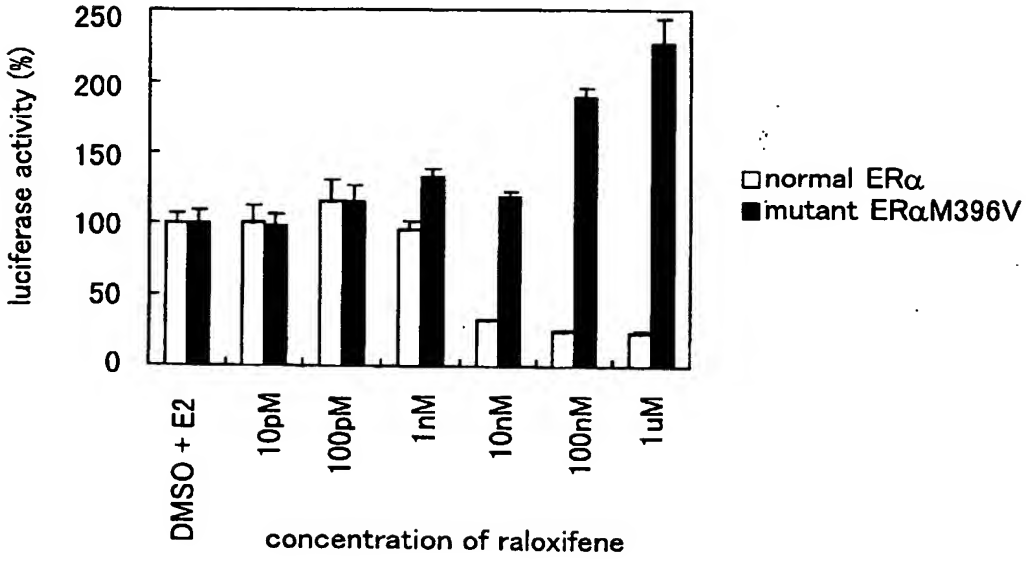

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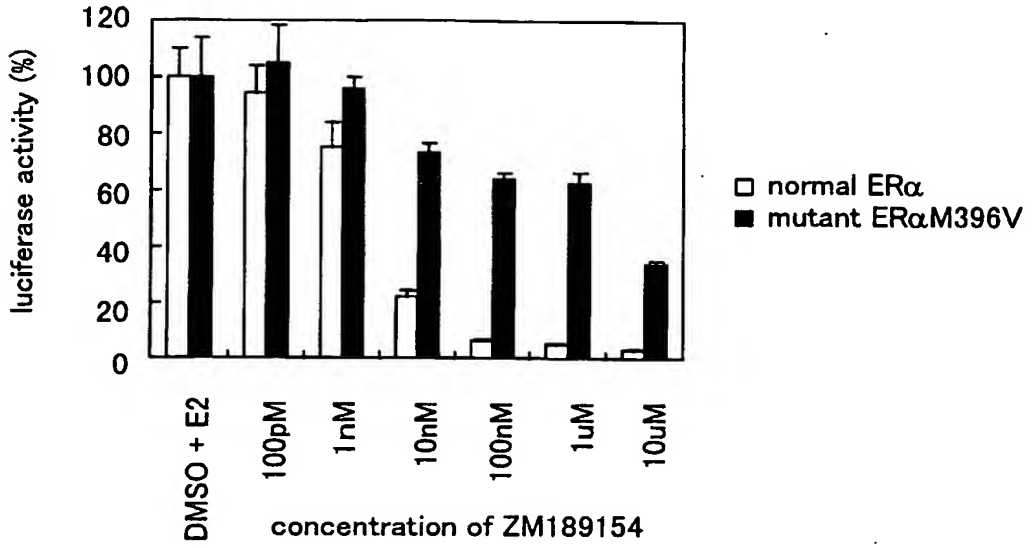

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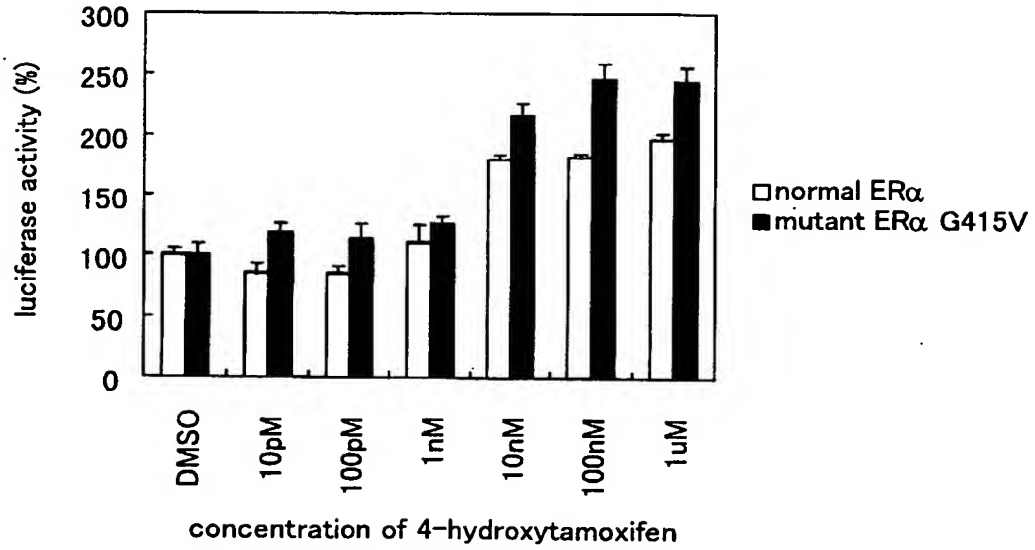

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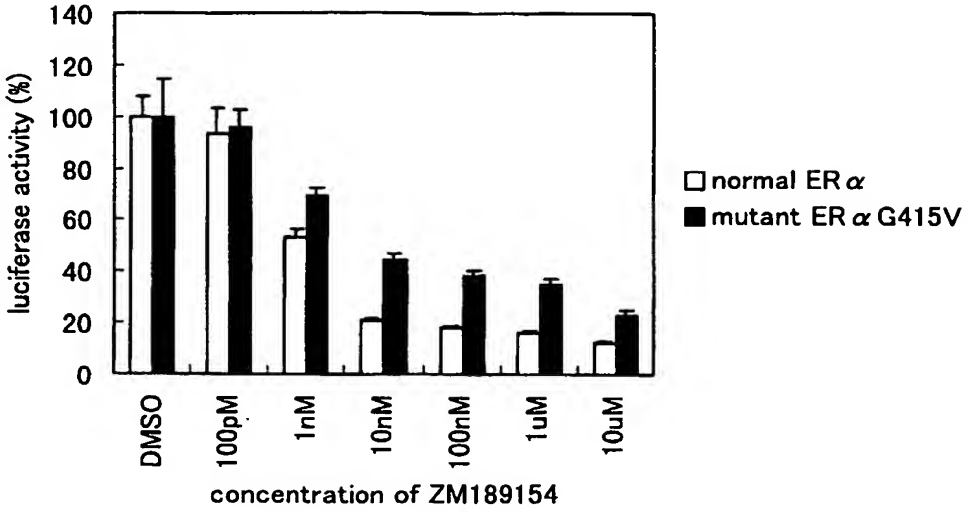

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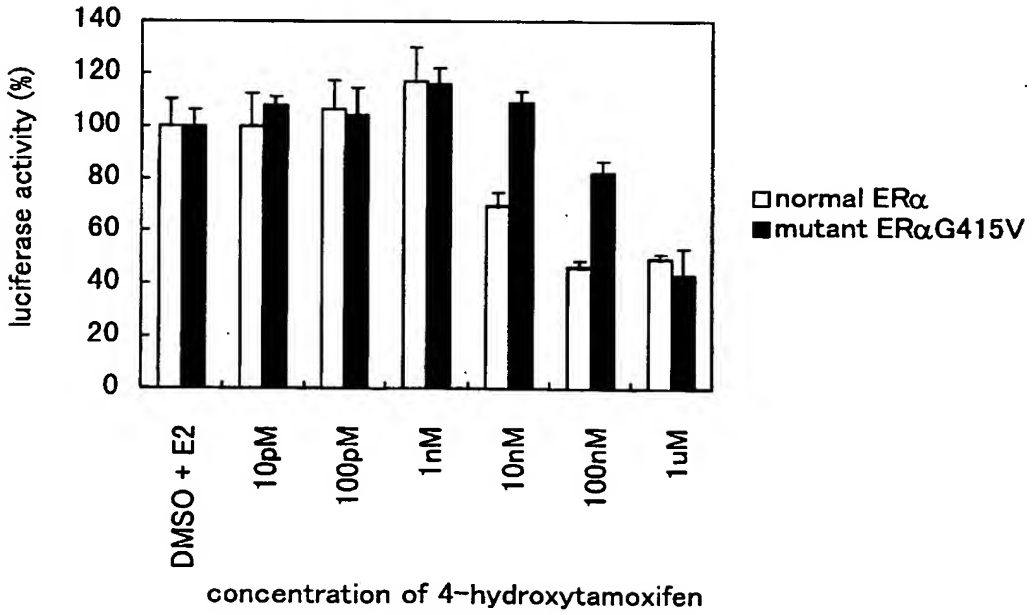

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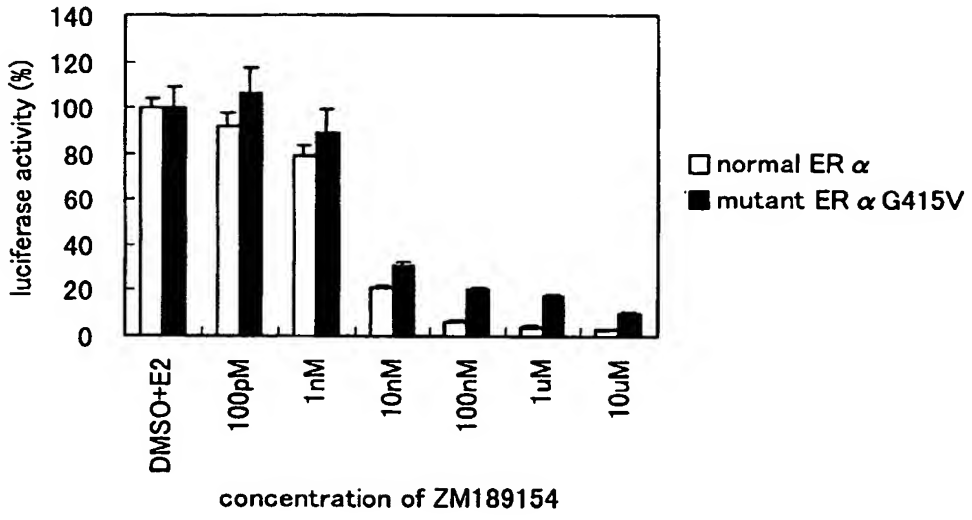

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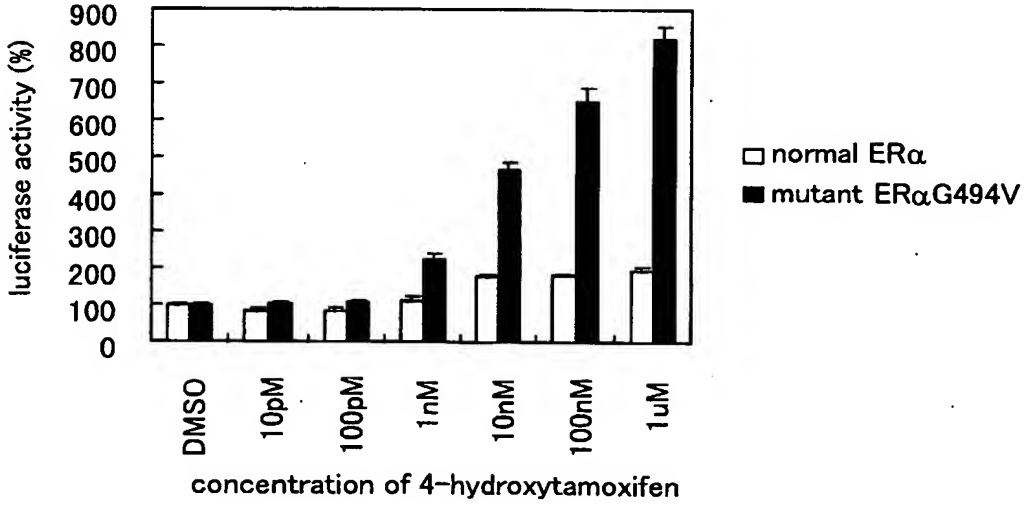

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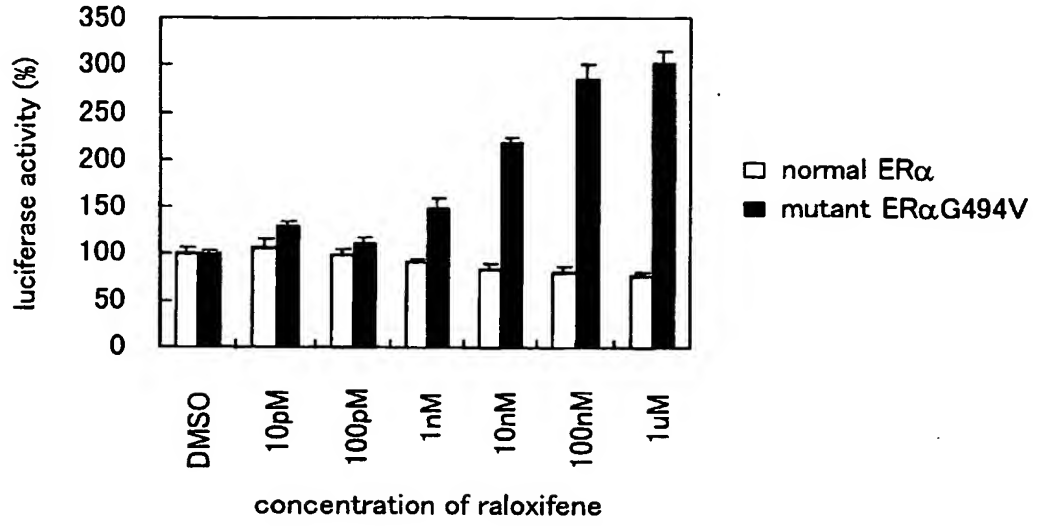

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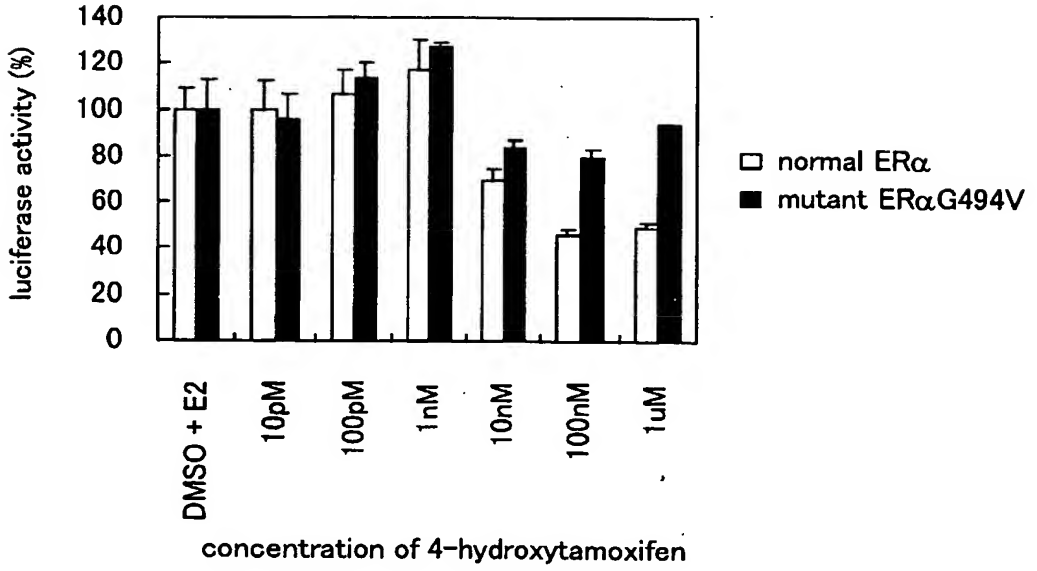

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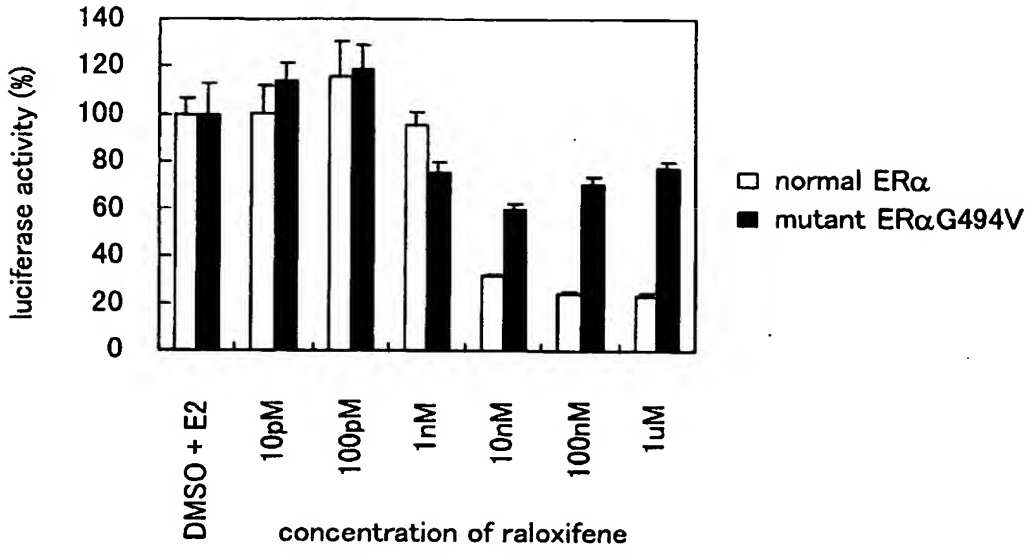

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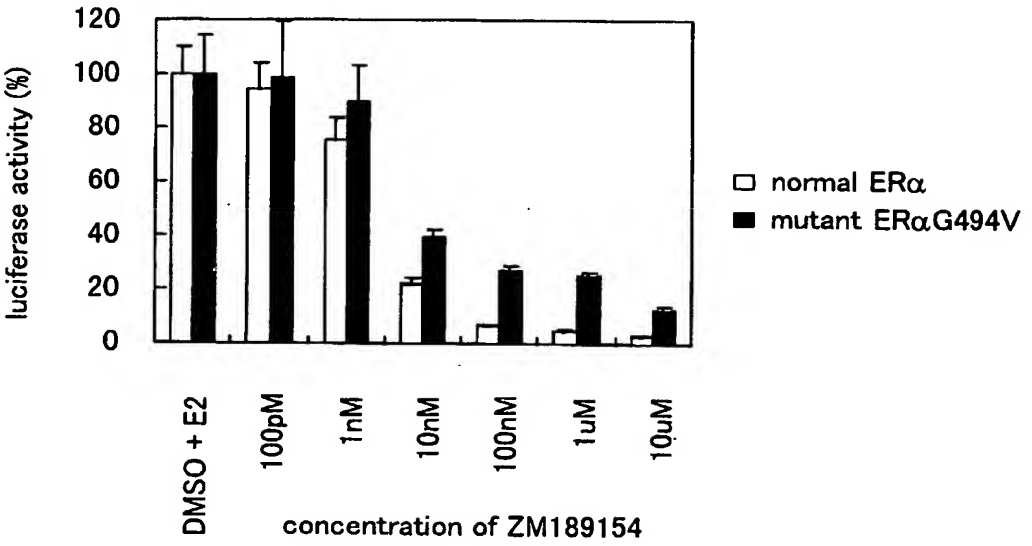

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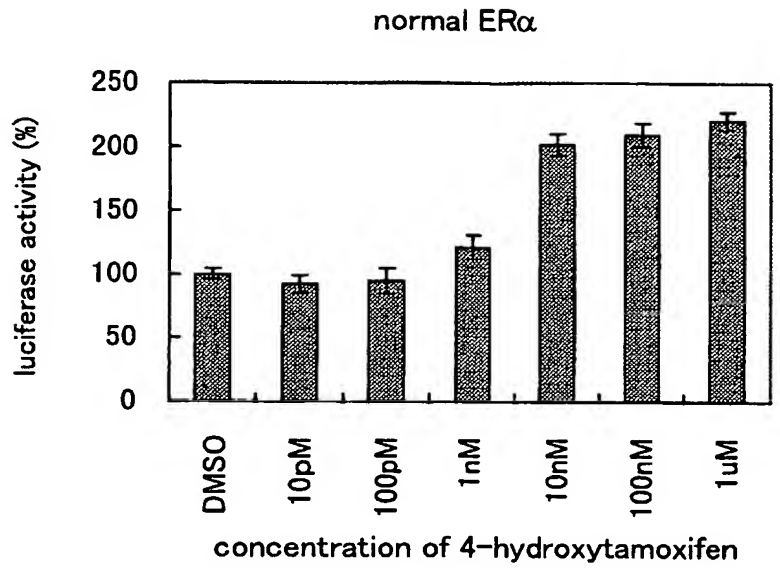

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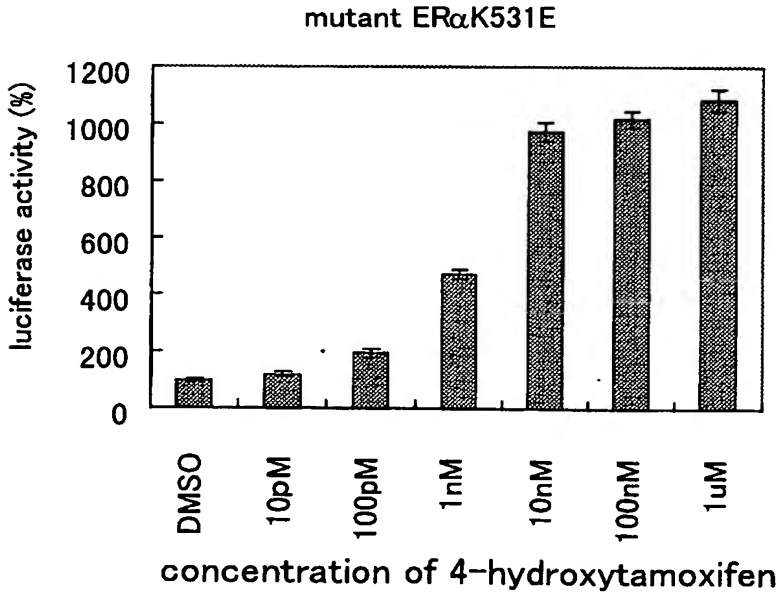

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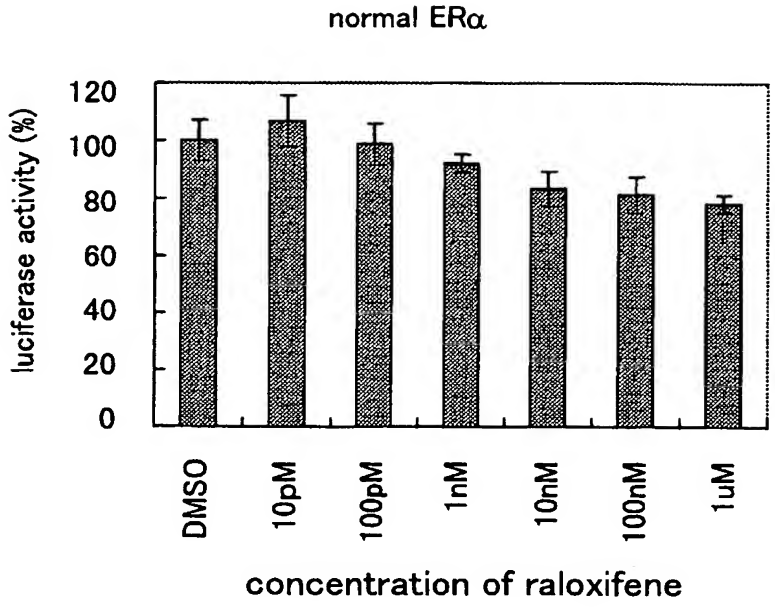

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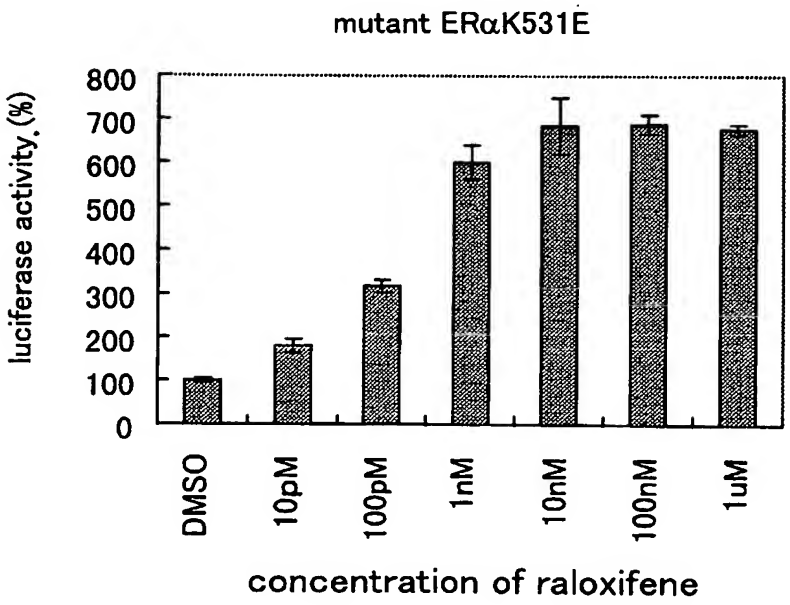

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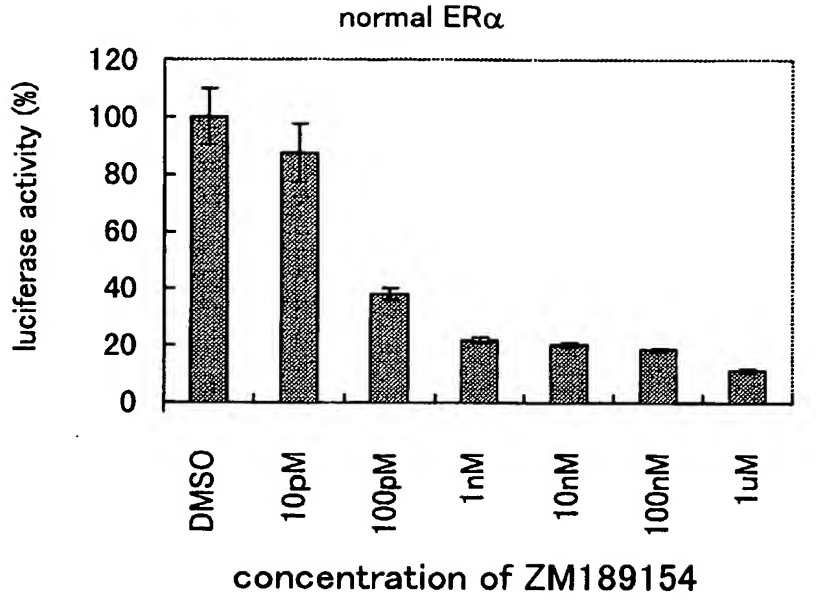

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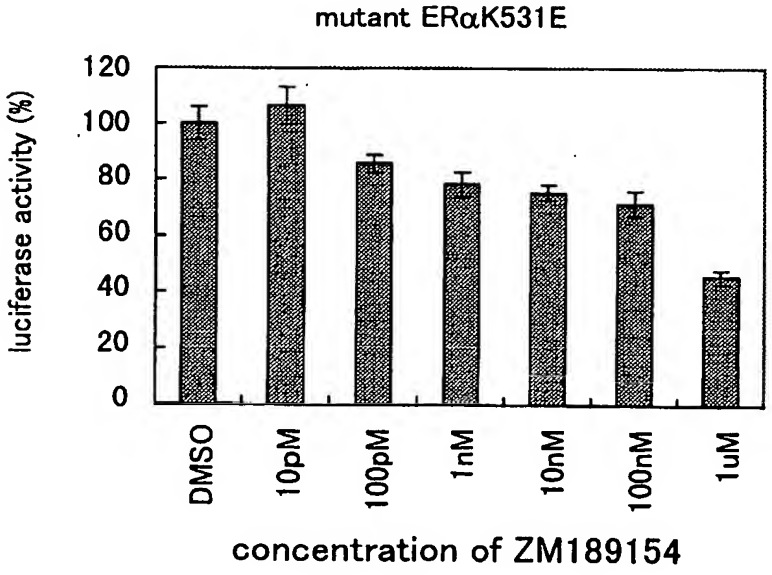

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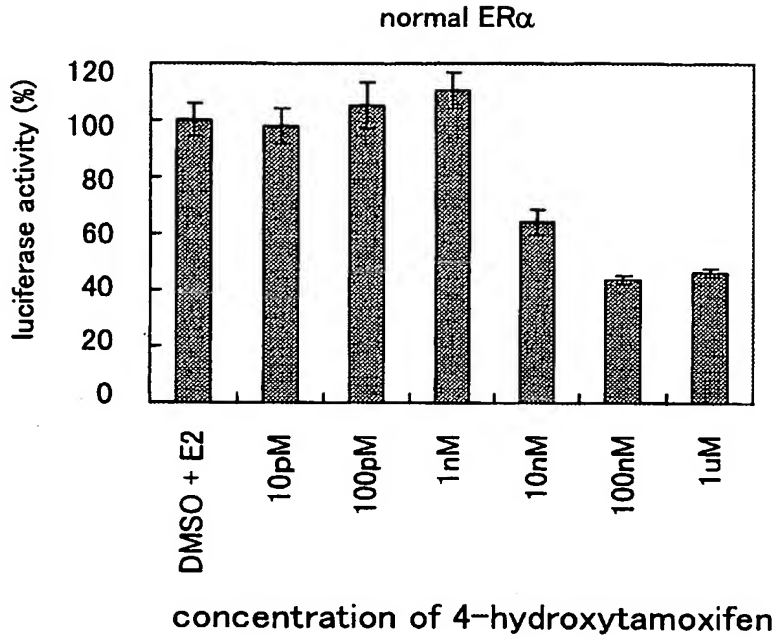

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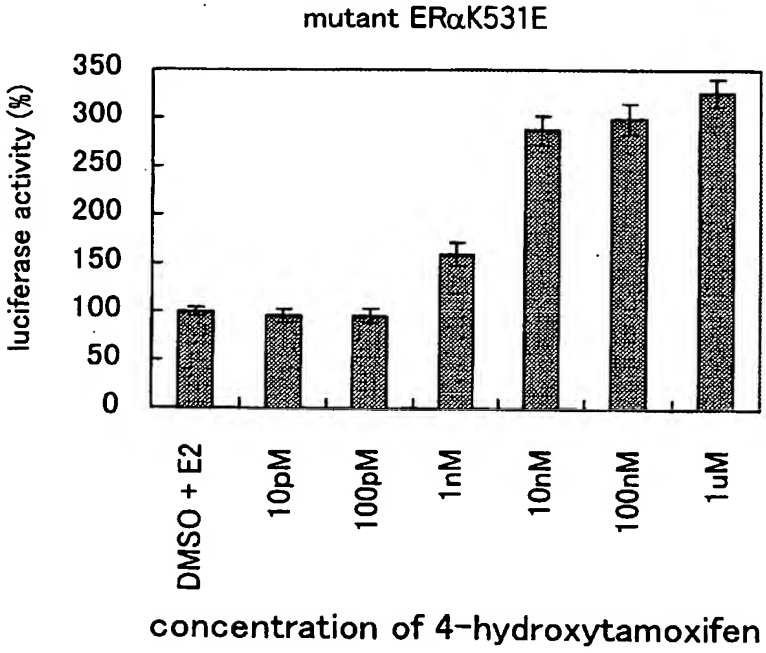

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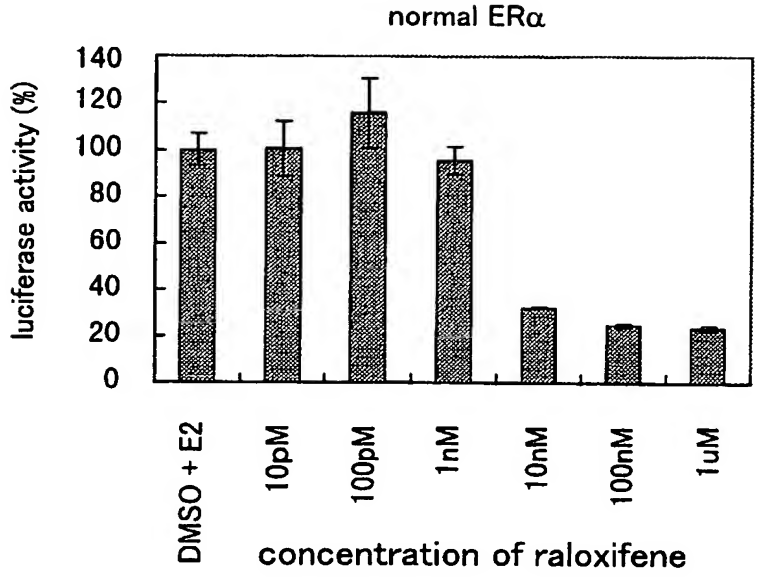

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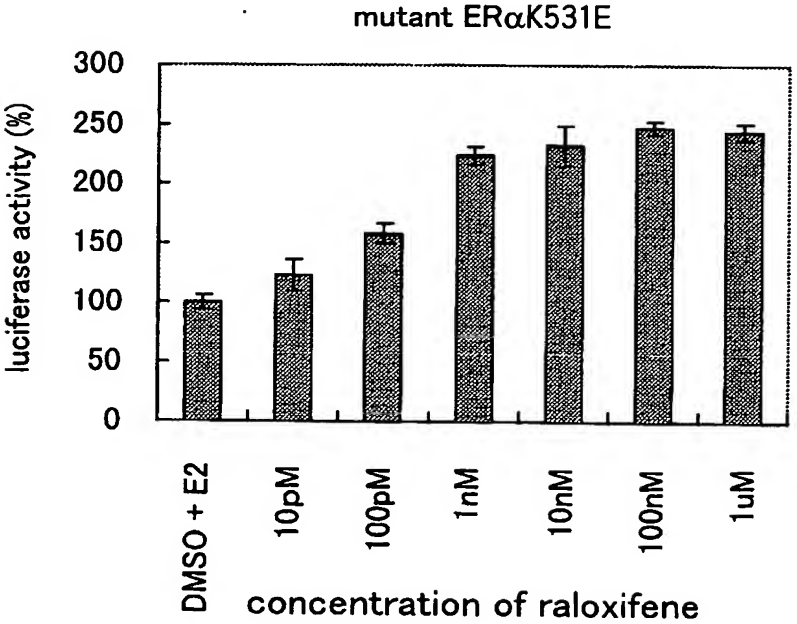

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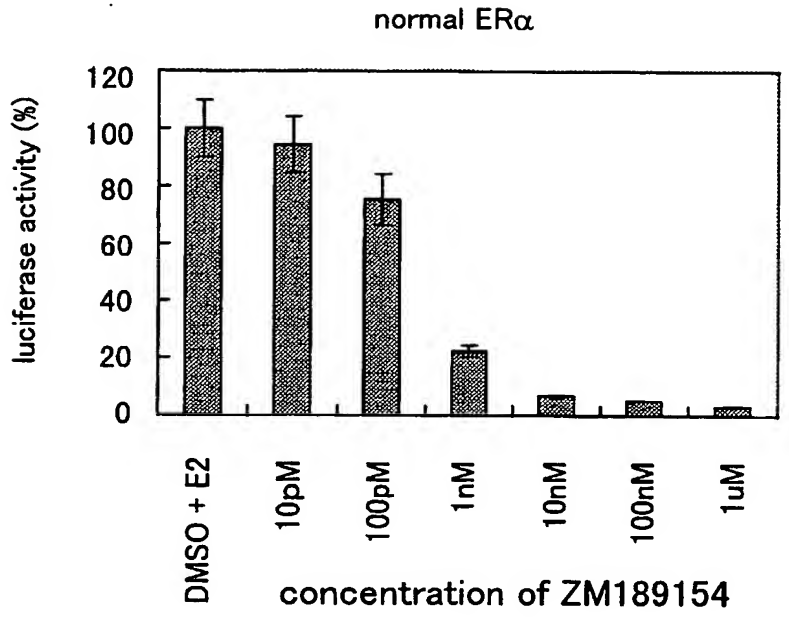

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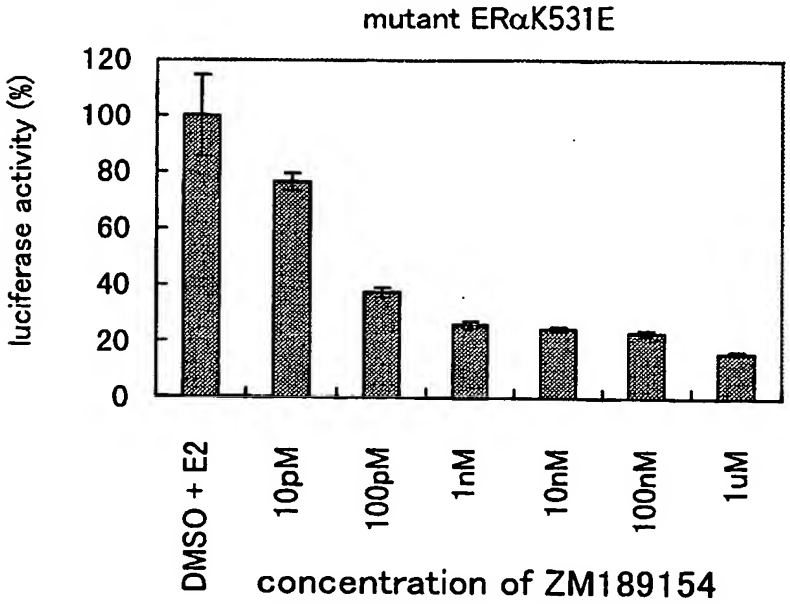

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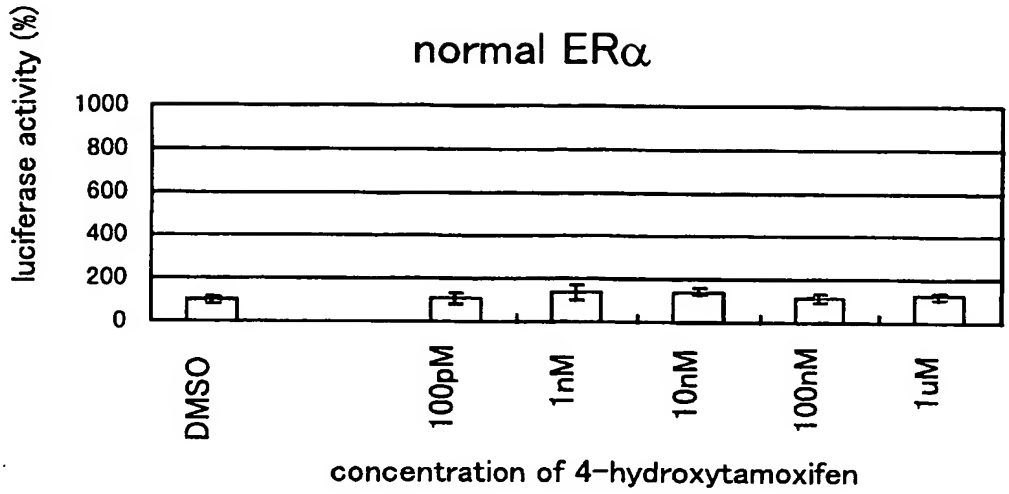

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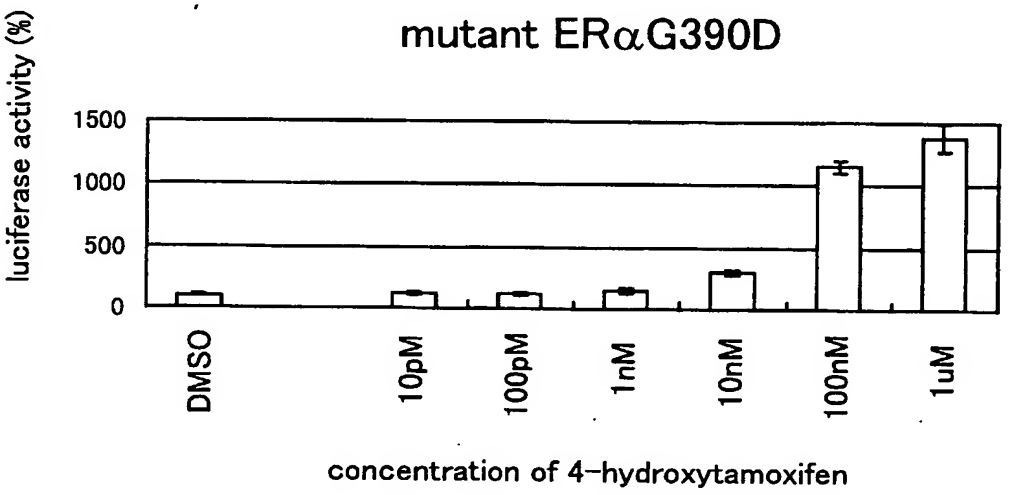

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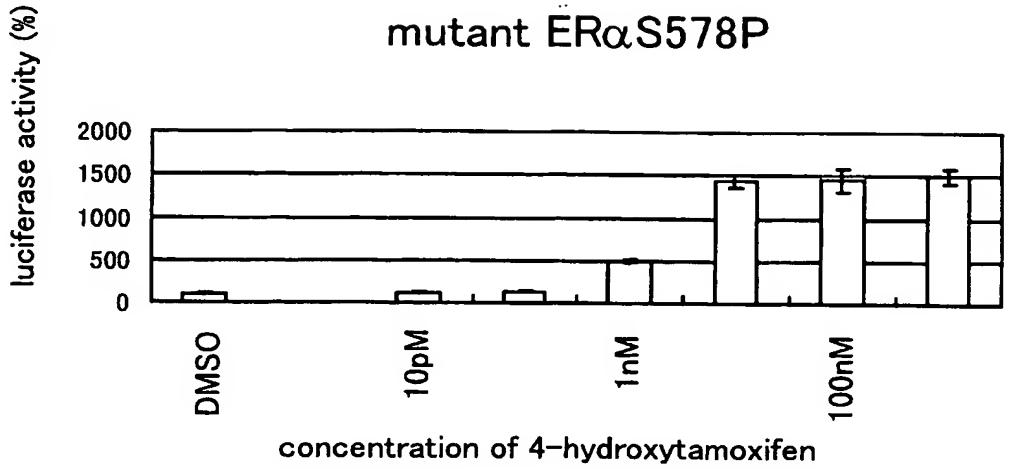

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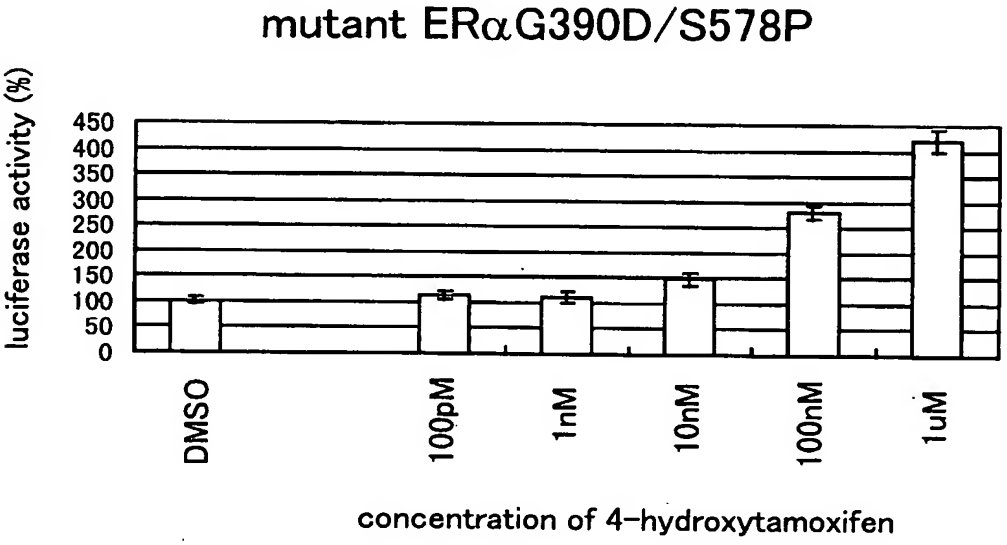

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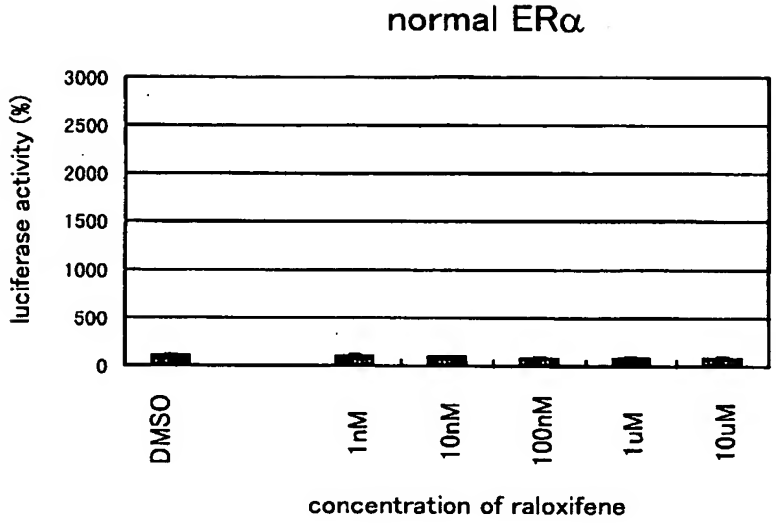

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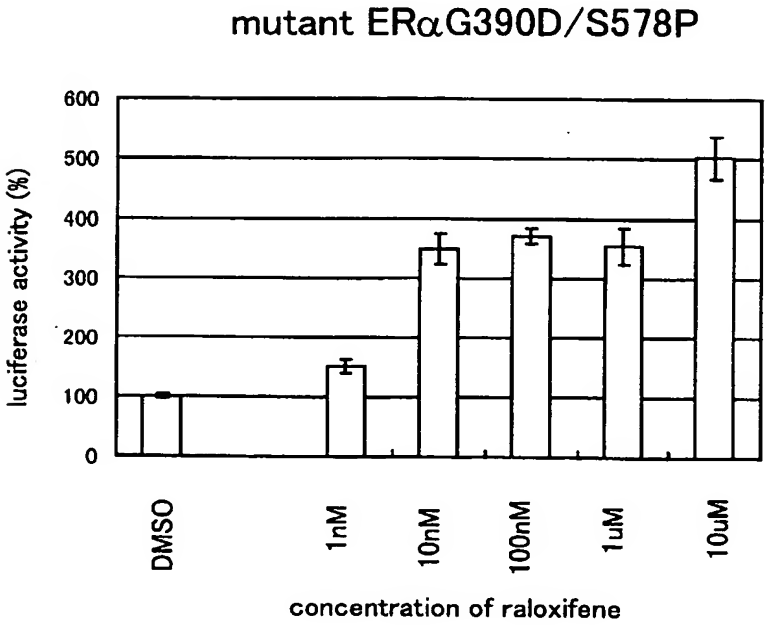

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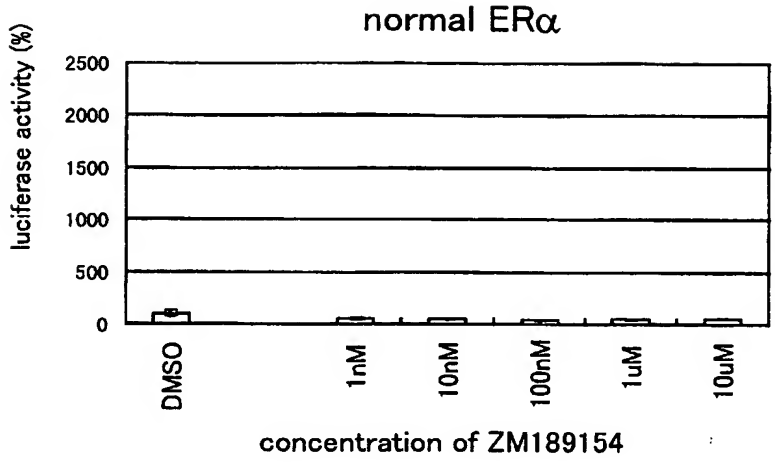

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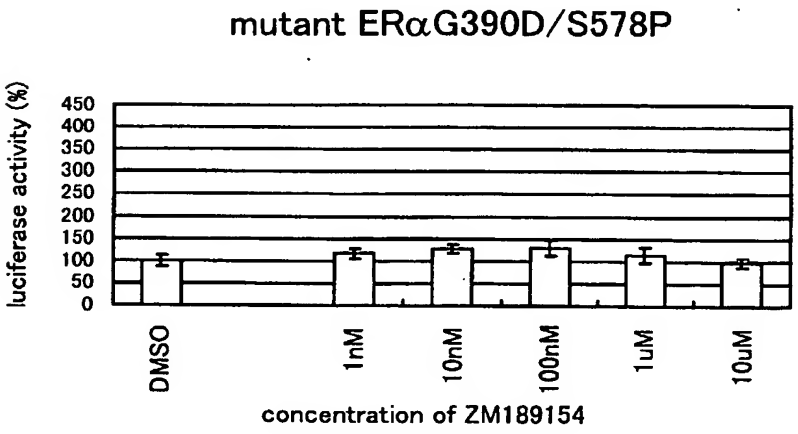

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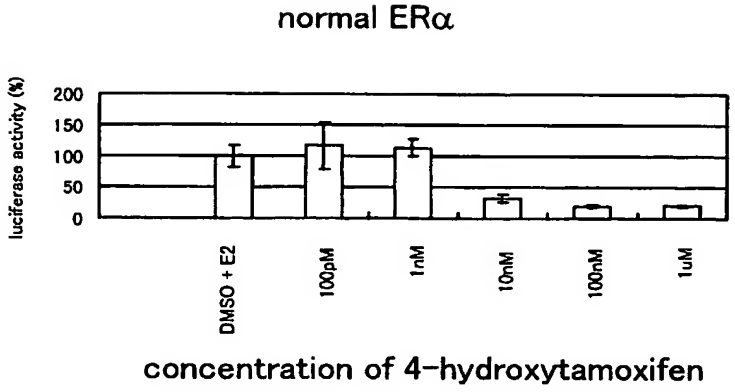

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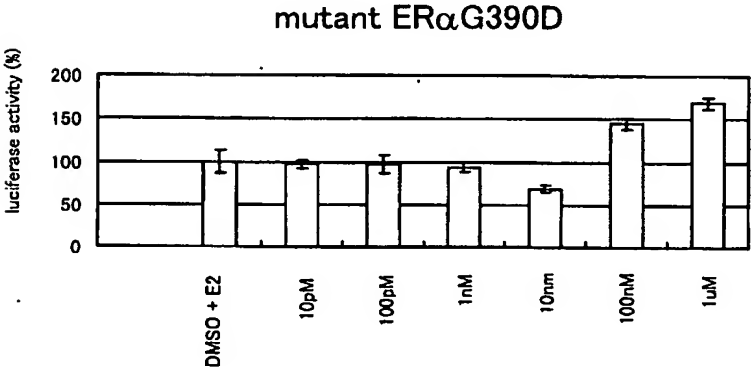

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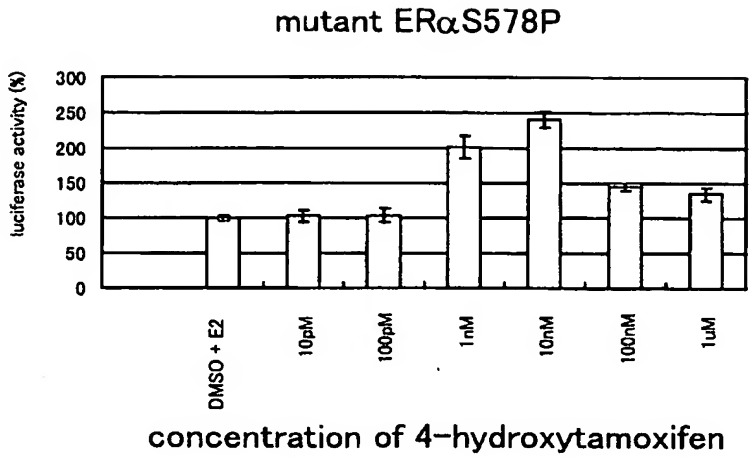

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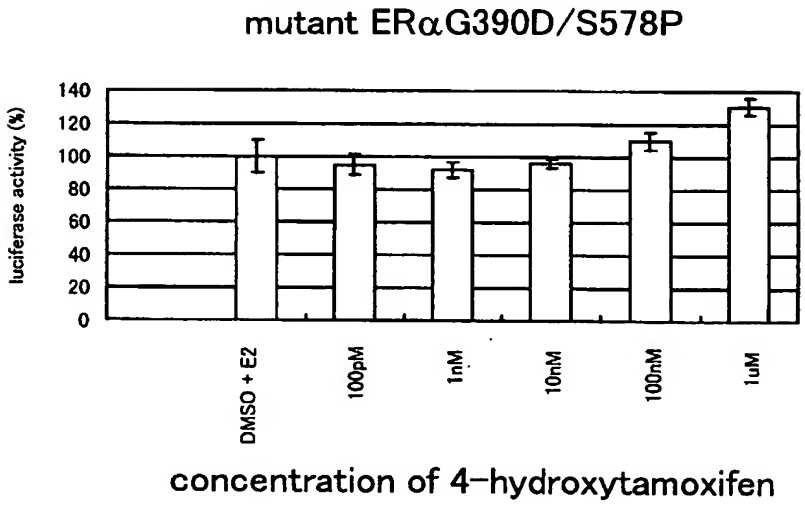

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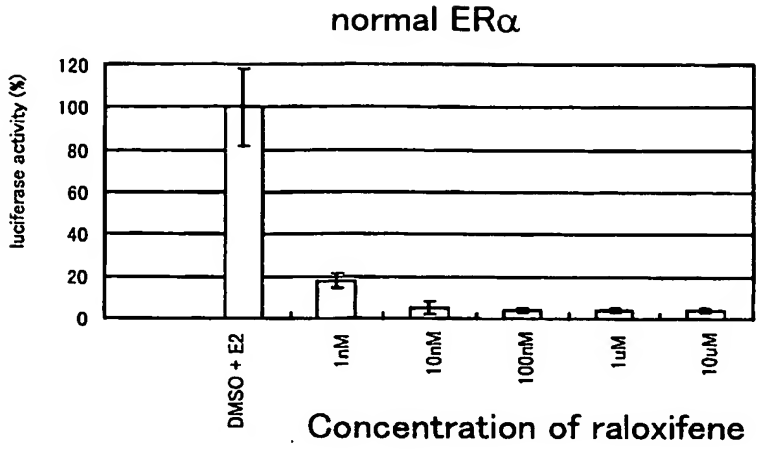

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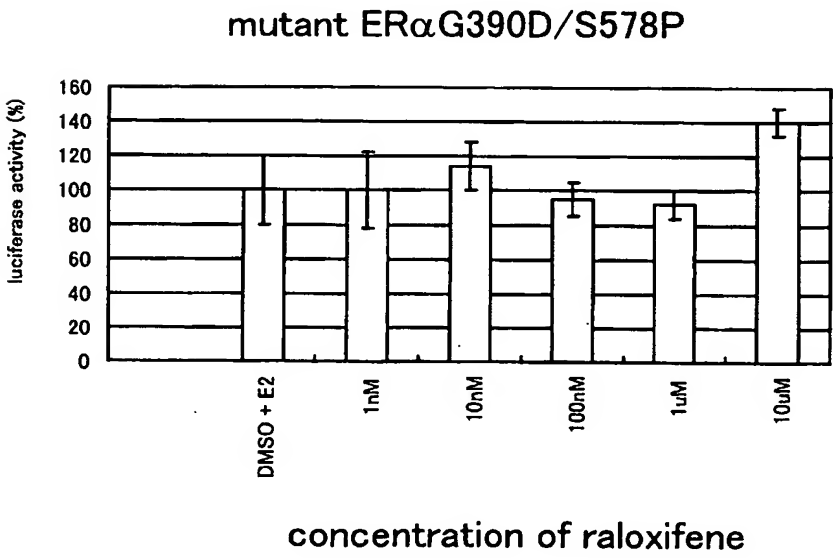

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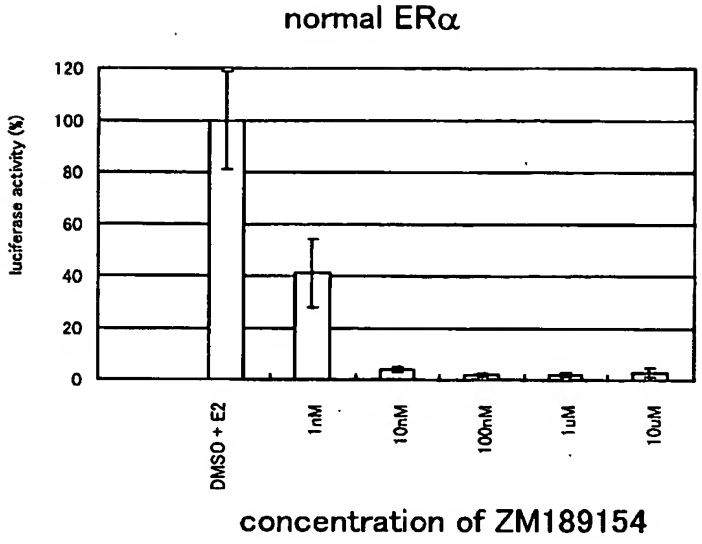

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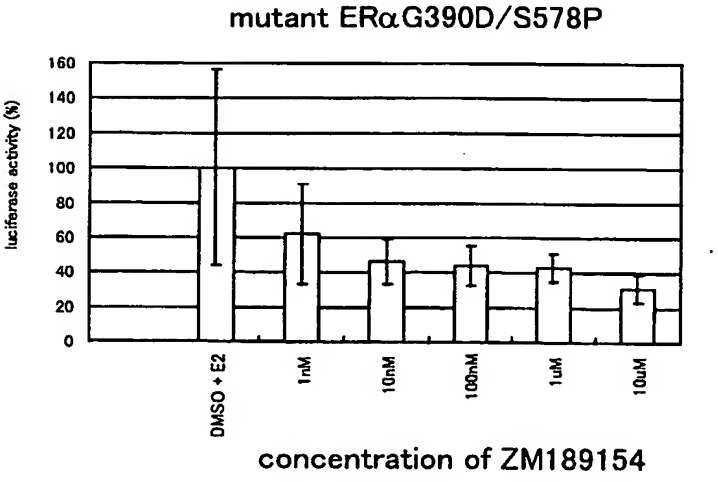


Figure 49

Dually transient reporter assay

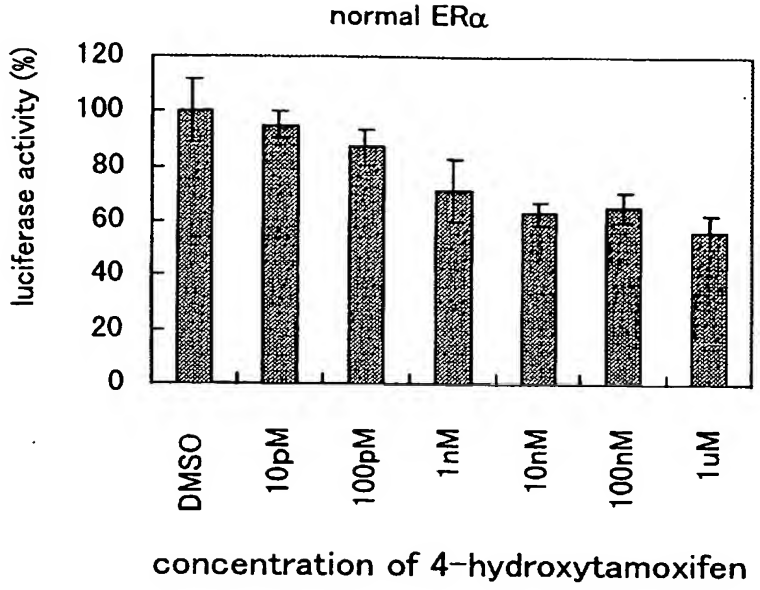


Figure 50

Dually transient reporter assay

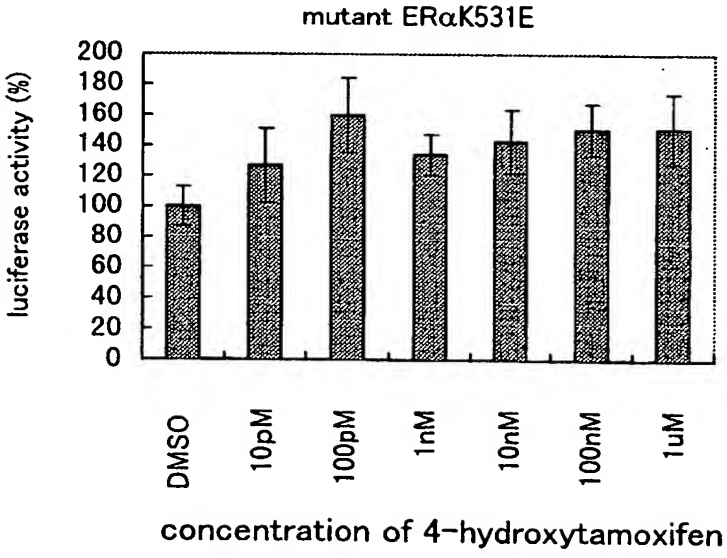


Figure 51

Dually transient reporter assay

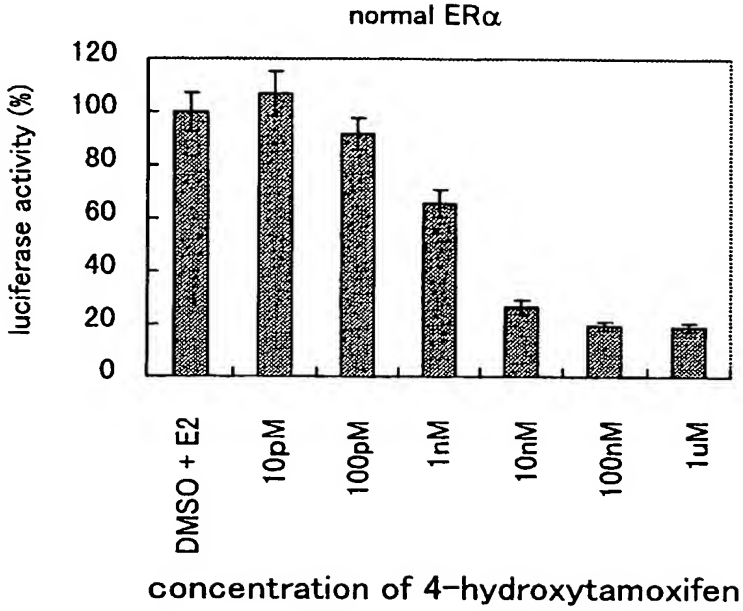
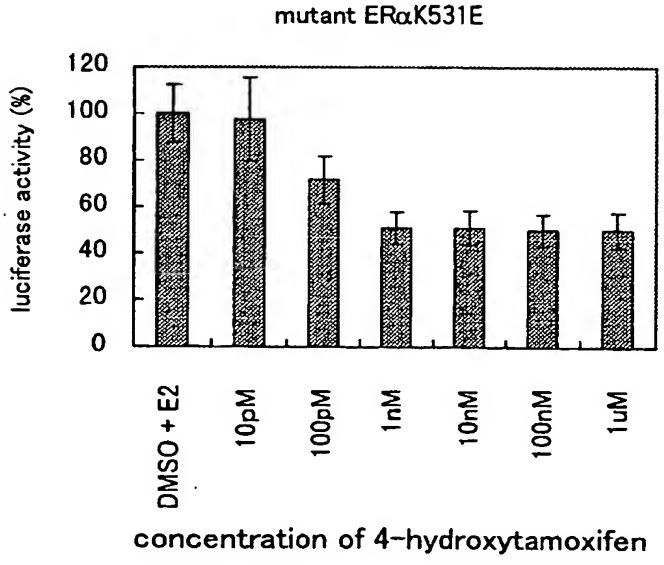


Figure 52

Dually transient reporter assay



SEQUENCE LISTING

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Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn
290 295 300

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Met Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr
485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
515 520 525

Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Pro Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

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<211> 595
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<213> Homo sapiens

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Gln Ile Gln Gly Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys
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Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Arg Asn
290 295 300

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Met Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr
485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
515 520 525

Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Pro Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

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<211> 595
<212> PRT
<213> Homo sapiens

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Gln Ile Gln Gly Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys
20 25 30

Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala

130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn
290 295 300

Ser Leu Ala Leu Phe Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro

325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Met Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr
485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met

515 520 525

Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Pro Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

<210> 4
<211> 595
<212> PRT
<213> Homo sapiens

<400> 4
Met Thr Met Thr Leu His Thr Lys Ala Ser Gly Met Ala Leu Leu His
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Gln Ile Gln Gly Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys
20 25 30

Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn
290 295 300

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Asp Leu Val Trp Arg Ser Met Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr
485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
515 520 525

Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Ser Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

<210> 5
<211> 595
<212> PRT
<213> Homo sapiens

<400> 5

Met Thr Met Thr Leu His Thr Lys Ala Ser Gly Met Ala Leu Leu His
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Gln Ile Gln Gly Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys
20 25 30

Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn
290 295 300

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Val Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr
485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
515 520 525

Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Pro Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

<210> 6
<211> 595
<212> PRT
<213> Homo sapiens

<400> 6
Met Thr Met Thr Leu His Thr Lys Ala Ser Gly Met Ala Leu Leu His
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Gln Ile Gln Gly Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys
20 25 30

Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val

115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn
290 295 300

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu

305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Met Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Val Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr
485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser

500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
515 520 525

Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Pro Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

<210> 7
<211> 595
<212> PRT
<213> Homo sapiens

<400> 7
Met Thr Met Thr Leu His Thr Lys Ala Ser Gly Met Ala Leu Leu His
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Gln Ile Gln Gly Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys
20 25 30

Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn
290 295 300

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Met Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Val Leu Thr
485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
515 520 525

Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Pro Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

<210> 8
<211> 595

<212> PRT
<213> Homo sapiens

<400> 8
Met Thr Met Thr Leu His Thr Lys Ala Ser Gly Met Ala Leu Leu His
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Gln Ile Gln Gly Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys
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Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn
290 295 300

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Val Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr
485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
515 520 525

Lys Cys Glu Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Ser Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

<210> 9
<211> 595
<212> PRT
<213> Homo sapiens

<400> 9
Met Thr Met Thr Leu His Thr Lys Ala Ser Gly Met Ala Leu Leu His
1 5 10 15

Gln Ile Gln Gly Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys
20 25 30

Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His

100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn

290 295 300

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Met Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr

485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
515 520 525

Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Pro Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

<210> 10
<211> 595
<212> PRT
<213> Homo sapiens

<400> 10
Met Thr Met Thr Leu His Thr Lys Ala Ser Gly Met Ala Leu Leu His
1 5 10 15

Gln Ile Gln Gly Asn Glu Leu Glu Pro Leu Asn Arg Pro Gln Leu Lys
20 25 30

Ile Pro Leu Glu Arg Pro Leu Gly Glu Val Tyr Leu Asp Ser Ser Lys
35 40 45

Pro Ala Val Tyr Asn Tyr Pro Glu Gly Ala Ala Tyr Glu Phe Asn Ala
50 55 60

Ala Ala Ala Ala Asn Ala Gln Val Tyr Gly Gln Thr Gly Leu Pro Tyr
65 70 75 80

Gly Pro Gly Ser Glu Ala Ala Ala Phe Gly Ser Asn Gly Leu Gly Gly
85 90 95

Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Pro Leu Met Leu Leu His
100 105 110

Pro Pro Pro Gln Leu Ser Pro Phe Leu Gln Pro His Gly Gln Gln Val
115 120 125

Pro Tyr Tyr Leu Glu Asn Glu Pro Ser Gly Tyr Thr Val Arg Glu Ala
130 135 140

Gly Pro Pro Ala Phe Tyr Arg Pro Asn Ser Asp Asn Arg Arg Gln Gly
145 150 155 160

Gly Arg Glu Arg Leu Ala Ser Thr Asn Asp Lys Gly Ser Met Ala Met
165 170 175

Glu Ser Ala Lys Glu Thr Arg Tyr Cys Ala Val Cys Asn Asp Tyr Ala
180 185 190

Ser Gly Tyr His Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe
195 200 205

Phe Lys Arg Ser Ile Gln Gly His Asn Asp Tyr Met Cys Pro Ala Thr
210 215 220

Asn Gln Cys Thr Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys
225 230 235 240

Arg Leu Arg Lys Cys Tyr Glu Val Gly Met Met Lys Gly Gly Ile Arg
245 250 255

Lys Asp Arg Arg Gly Gly Arg Met Leu Lys His Lys Arg Gln Arg Asp
260 265 270

Asp Gly Glu Gly Arg Gly Glu Val Gly Ser Ala Gly Asp Met Arg Ala
275 280 285

Ala Asn Leu Trp Pro Ser Pro Leu Met Ile Lys Arg Ser Lys Lys Asn
290 295 300

Ser Leu Ala Leu Ser Leu Thr Ala Asp Gln Met Val Ser Ala Leu Leu
305 310 315 320

Asp Ala Glu Pro Pro Ile Leu Tyr Ser Glu Tyr Asp Pro Thr Arg Pro
325 330 335

Phe Ser Glu Ala Ser Met Met Gly Leu Leu Thr Asn Leu Ala Asp Arg
340 345 350

Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val Pro Gly Phe Val
355 360 365

Asp Leu Thr Leu His Asp Gln Val His Leu Leu Glu Cys Ala Trp Leu
370 375 380

Glu Ile Leu Met Ile Asp Leu Val Trp Arg Ser Met Glu His Pro Gly
385 390 395 400

Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg Asn Gln Gly Lys
405 410 415

Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu Leu Ala Thr Ser
420 425 430

Ser Arg Phe Arg Met Met Asn Leu Gln Gly Glu Glu Phe Val Cys Leu
435 440 445

Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr Phe Leu Ser Ser
450 455 460

Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His Arg Val Leu Asp
465 470 475 480

Lys Ile Thr Asp Thr Leu Ile His Leu Met Ala Lys Ala Gly Leu Thr
485 490 495

Leu Gln Gln Gln His Gln Arg Leu Ala Gln Leu Leu Leu Ile Leu Ser
500 505 510

His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu Tyr Ser Met
515 520 525

Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu Glu Met Leu
530 535 540

Asp Ala His Arg Leu His Ala Pro Thr Ser Arg Gly Gly Ala Ser Val
545 550 555 560

Glu Glu Thr Asp Gln Ser His Leu Ala Thr Ala Gly Ser Thr Ser Ser
565 570 575

His Pro Leu Gln Lys Tyr Tyr Ile Thr Gly Glu Ala Glu Gly Phe Pro
580 585 590

Ala Thr Val
595

<210> 11
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 11
cctgcgggga cacggtctgc accctgcccg cggcc 35

<210> 12
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 12
cagggagctc tcagactgtg gcagggaaac cctct 35

<210> 13
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 13
atgatcaaac gctctaagag gaacagcctg gccttgtccc tgacggccg 49

<210> 14
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 14
ggacaaggcc aggctgttcc tcttagagcg tttgatcatg agcgggctt 49

<210> 15
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 15
aagaacagcc tggccttgtt cctgacggcc gaccagatgg tcagtgcct 49

<210> 16
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 16
catctggtcg gccgtcagga acaaggccag gctgttcttc ttagagcgt 49

<210> 17
<211> 44
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 17
gctagagatc ctgatgattg atctcgtctg gcgctccatg gagc 44

<210> 18
<211> 44
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 18
gctccatgga gcgccagacg agatcaatca tcaggatctc tagc 44

<210> 19
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 19
tggtctcgtc tggcgctccg tggagcaccc agggaagcta ctgtttgct 49

<210> 20
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 20
agcaaacagt agcttccctg ggtgctccac ggagcgccag acgagacca 49

<210> 21
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 21
ctcttggaca ggaaccaggt aaaatgtgta gagggcatgg tggagatct 49

<210> 22
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 22
catgccctct acacatttta cctggttcct gtccaagagc aagttagga 49

<210> 23
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 23
ccacctgatg gccaaggcag tcctgaccct gcagcagcag cagcagcac 49

<210> 24
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 24
ggtgctgctg ctgcagggtc aggactgcct tggccatcag gtggatcaa 49

<210> 25
<211> 44
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 25
atctgtacag catgaagtgc gagaacgtgg tgcccctcta tgac 44

<210> 26
<211> 44
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 26
gtcatagagg ggcaccacgt tctcgcactt catgctgtac agat 44

<210> 27
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 27
gcgggctcta cttcatcgca tcccttgcaa aagtattaca tcacg 45

<210> 28
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide for mutagenesis

<400> 28
cgtgatgtaa tacttttgca agggatgcga tgaagtagag cccgc 45

<210> 29
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 29
tggagacatg agagctgcca 20

<210> 30
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 30
acctttggcc aagcccgctc 20

<210> 31
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 31
ccgctcatga tcaaacgctc 20

<210> 32
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 32
agggcagggg tgaagtgggg 20

<210> 33
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 33
ccaacctttg gccaagcccg 20

<210> 34
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 34
tactcggaat agagtatggg 20

<210> 35
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 35
ggctcagcat ccaacaaggc 20

<210> 36
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 36
tgaccatctg gtcggccgtc 20

<210> 37
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 37
aagggtctgg taggatcata 20

<210> 38
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 38
catctggtcg gccgtcaggg 20

<210> 39
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 39
agagctgcca acctttggcc 20

<210> 40
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 40
aagcccgctc atgatcaaac 20

<210> 41
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 41
gctctaagaa gaacagcctg 20

<210> 42
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 42
agacatgaga gctgccaacc 20

<210> 43
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 43
ggccaagccc gctcatgatc 20

<210> 44
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 44
agcttcactg aagggtctgg 20

<210> 45
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 45
taggatcata ctcggaatag 20

<210> 46
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 46
agtatggggg gctcagcatc 20

<210> 47
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 47
caacaaggca ctgaccatct 20

<210> 48
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 48
aagggtctgg taggatcata 20

<210> 49
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 49
gagctggttc acatgatcaa 20

<210> 50
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 50
tgggcgaaga gggtgccagg 20

<210> 51
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 51
ttgtggattt gaccctccat 20

<210> 52
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 52
atcaggtcca ccttctagaa 20

<210> 53
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 53
gtgcctggct agagatcctg 20

<210> 54
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 54
tgggtgctcc atggagcgcc 20

<210> 55
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 55
ttaggagcaa acagtagctt 20

<210> 56
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 56
tggttcctgt ccaagagca 19

<210> 57
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 57
tccaccatgc cctctacaca 20

<210> 58
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 58
agccagcagc atgtcgaaga 20

<210> 59
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 59
ggctttggtg atttgaccct 20

<210> 60
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 60
ccatgatcag gtccaccttc 20

<210> 61
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 61
tagaatgtgc ctggctagag 20

<210> 62
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 62
gctggttcac atgatcaact 20

<210> 63
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 63
gccaggcttt gtggatttga 20

<210> 64
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 64
atgatgtagc cagcaggtcg 20

<210> 65
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 65
gccctctaca cattttccct 20

<210> 66
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 66
aagagcaagt taggagcaaa 20

<210> 67
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 67
ggatcaaagt gtctgtgatc 20

<210> 68
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 68
tctccaccat gccctctaca 20

<210> 69
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 69
ggtctcgtct ggcgctccat 20

<210> 70
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 70
ggagcaccca gtgaagctac 20

<210> 71
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 71
tgtttgctcc taacttggac 20

<210> 72
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 72
tcctgatgat tggtctcgtc 20

<210> 73
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 73
cacccagtga agctactgtt 20

<210> 74
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 74
ttcatcatgc ggaaccgaga 20

<210> 75
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 75
gatgtagcca gcagcatgtc 20

<210> 76
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 76
aagatctcca ccatgcccct 20

<210> 77
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 77
ctccaccatg cccctctaca 20

<210> 78
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 78
atgtcgaaga tctccaccat 20

<210> 79
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 79
agagaaggac catatccacc 20

<210> 80
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 80
gagtcctgga caagatcaca 20

<210> 81
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 81
gacactttga tccacctgat 20

<210> 82
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 82
ccctgaagtc tctggaagag 20

<210> 83
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 83
acaagatcac agacactttg 20

<210> 84
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 84
tgtgcctgat gtgggagagg 20

<210> 85
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 85
atgaggagga gctgggccag 20

<210> 86
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 86
ccgctggtgc tgctgctgca 20

<210> 87
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 87
gctgggccag ccgctggtgc 20

<210> 88
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 88
cctttgttac tcatgtgcct 20

<210> 89
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 89
aggcacatga gtaacaaagg 20

<210> 90
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Designed oligonucleotide primer for PCR

<400> 90
catggagcat ctgtacagca 20

<210> 91
<211> 20
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<210> 119
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oligonucleotide probe for Southern hybridization

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<210> 120
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<210> 121
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oligonucleotide probe for Southern hybridization

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oligonucleotide probe for Southern hybridization

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<210> 123
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oligonucleotide probe for Southern hybridization

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<210> 124
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oligonucleotide probe for Southern hybridization

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oligonucleotide probe for Southern hybridization

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<210> 127
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<210> 128
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oligonucleotide probe for Southern hybridization

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<210> 129
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oligonucleotide probe for Southern hybridization

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oligonucleotide probe for Southern hybridization

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<210> 133
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<210> 134
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<210> 135
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<210> 136
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oligonucleotide probe for Southern hybridization

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<210> 138
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<210> 139
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oligonucleotide probe for Southern hybridization

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<210> 140
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oligonucleotide probe for Southern hybridization

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<210> 141
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oligonucleotide probe for Southern hybridization

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<210> 142
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<210> 143
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<210> 144
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<210> 145
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oligonucleotide probe for Southern hybridization

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<210> 146
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oligonucleotide probe for Southern hybridization

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<210> 147
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<210> 148
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<210> 149
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<210> 150
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<210> 155
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aatttatgct acaacaaggc aaggc 25

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ggagtggcac cttccagggt caag 24

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oligonucleotide for synthesis

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<210> 163
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oligonucleotide for synthesis

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<210> 168
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<220>
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<400> 168
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<210> 169
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<210> 170
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<210> 171
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<210> 173
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<210> 174
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<210> 176
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<400> 176
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<210> 177
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<210> 178
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<400> 178
cccagccacc atggaagtgc agttagggct gggaagggtc 40

<210> 179
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gggtggggaa atagggtttc caatgcttca ctggg 35

<210> 180
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<210> 185
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<210> 186
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<210> 190
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<210> 191
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oligonucleotide primer for PCR

<400> 195
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oligonucleotide primer for PCR

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<210> 197
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<400> 197
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<220>
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<211> 35
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<213> Artificial Sequence

<220>
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<400> 203
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<212> DNA
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<220>
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<210> 205
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<210> 206
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<220>
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<210> 207
<211> 33
<212> DNA
<213> Artificial Sequence

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<400> 207
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<212> DNA
<213> Artificial Sequence

<220>
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<223> Description of Artificial Sequence :Designed oligonucleotide for synthesis

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<210> 210
<211> 30
<212> DNA
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<220>
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<210> 211
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<210> 212
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<220>
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence :Designed oligonucleotide for synthesis

<400> 213
tcaggtcaca ggtcatg 17

INTERNATIONAL SEARCH REPORT

International Application No
PCT/JP 00/08553

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/72 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, STRAND, WPI Data, PAJ, MEDLINE, BIOTECHNOLOGY ABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BERRY M ET AL: "ROLE OF THE TWO ACTIVATING DOMAINS OF THE ESTROGEN RECEPTOR IN THE CELL-TYPE AND PROMOTER-CONTEXT DEPENDENT AGONISTIC ACTIVITY OF THE ANTI-ESTROGEN 4 HYDROXYTAMOXIFEN" EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 9, no. 9, 1990, pages 2811-2818, XP002009521 ISSN: 0261-4189 cited in the application	1,3,4,9, 10,32-46
X	page 2812, column 1, paragraph 2 -page 2813, column 1, paragraph 2; figure 1 page 2817, column 1, paragraph 2 -column 2	1,3,4,9, 10,32-46
A	page 2817, column 1, line 15, paragraph 1 - line 21	47

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 April 2001	11/05/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHWARTZ J A ET AL: "Neutral mutations to three acidic AF2 residues in the mouse estrogen receptor confer agonist activity to A-ring isomers of estradiol." JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY, vol. 62, no. 2-3, June 1997 (1997-06), pages 173-184, XP000926278 ISSN: 0960-0760 page 176; figure 3 ---	1
X	MCDONNELL D P ET AL: "NUCLEAR HORMONE RECEPTORS AS TARGETS FOR NEW DRUG DISCOVERY" BIO/TECHNOLOGY, vol. 11, 1 November 1993 (1993-11-01), pages 1256-1261, XP002018219 NEW YORK US ISSN: 0733-222X page 1259, column 2, last paragraph -page 1260, column 1, paragraph 2; figure 5 ---	36-38
X	MONTANO MONICA M ET AL: "Human estrogen receptor ligand activity inversion mutants: Receptors that interpret antiestrogens as estrogens and estrogens as antiestrogens and discriminate among different antiestrogens." MOLECULAR ENDOCRINOLOGY, vol. 10, no. 3, 1996, pages 230-242, XP000926402 BETHESDA US ISSN: 0888-8809 abstract page 231, column 2, paragraph 2 -page 233, column 1, paragraph 2 ---	1,5
X	UMEKITA YOSHIHISA ET AL: "Estrogen receptor mutations and changes in estrogen receptor and progesterone receptor protein expression in metastatic or recurrent breast cancer." JAPANESE JOURNAL OF CANCER RESEARCH, vol. 89, no. 1, January 1998 (1998-01), pages 27-32, XP000926289 ISSN: 0910-5050 abstract page 27 -page 29 --- -/--	11-14, 19,27, 30,31, 50-52

INTERNATIONAL SEARCH REPORT

International Application No
PCT/JP 00/08553

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROODI NADY ET AL: "Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer." JOURNAL OF THE NATIONAL CANCER INSTITUTE (BETHESDA), vol. 87, no. 6, 1995, pages 446-451, XP000926290 ISSN: 0027-8874 abstract page 447 -page 449 ---	1-14,18, 26,50-52
A	ENGLAND GALE M ET AL: "Characterization of a point mutation in the hormone binding domain of the estrogen receptor from an 'estrogen independent' breast tumor." INTERNATIONAL JOURNAL OF ONCOLOGY, vol. 12, no. 5, May 1998 (1998-05), pages 981-986, XP000926285 ISSN: 1019-6439 abstract ---	1-31, 50-52
A	KATZENELLENBOGEN BENITA S ET AL: "Antiestrogens: Mechanisms and actions in target cells." JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY, vol. 53, no. 1-6, 1995, pages 387-393, XP000926403 ISSN: 0960-0760 the whole document ---	1
A	WO 87 05049 A (CALIFORNIA BIOTECHNOLOGY INC) 27 August 1987 (1987-08-27) the whole document -----	1-31

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-14, 22, 30, 31, 39-55 all partially and 15, 23 completely

An artificial cell comprising i) a chromosome with a reporter gene, which comprises an ERE, a TATA sequence and a reporter sequence and ii) a mutant ERalpha having an amino acid sequence shown in SEQ.ID.2; an isolated mutant ERalpha having said sequence; a polynucleotide encoding said mutant ERalpha; use of said cell in screening methods and methods for diagnosing the genotype or the phenotype of said mutant ERalpha.

2. Claims: 1-14, 22, 30, 31, 39-55 all partially and 16, 24 completely

An artificial cell comprising i) a chromosome with a reporter gene, which comprises an ERE, a TATA sequence and a reporter sequence and ii) a mutant ERalpha having an amino acid sequence shown in SEQ.ID.3; an isolated mutant ERalpha having said sequence; a polynucleotide encoding said mutant ERalpha; use of said cell in screening methods and methods for diagnosing the genotype or the phenotype of said mutant ERalpha.

3. Claims: 1-14, 22, 30, 31, 39-55 all partially and 17, 25 completely

An artificial cell comprising i) a chromosome with a reporter gene, which comprises an ERE, a TATA sequence and a reporter sequence and ii) a mutant ERalpha having an amino acid sequence shown in SEQ.ID.4; an isolated mutant ERalpha having said sequence; a polynucleotide encoding said mutant ERalpha; use of said cell in screening methods and methods for diagnosing the genotype or the phenotype of said mutant ERalpha.

4. Claims: 1-14, 22, 30, 31, 39-55 all partially and 18, 26 completely

An artificial cell comprising i) a chromosome with a reporter gene, which comprises an ERE, a TATA sequence and a reporter sequence and ii) a mutant ERalpha having an amino acid sequence shown in SEQ.ID.5; an isolated mutant ERalpha having said sequence; a polynucleotide encoding said mutant ERalpha; use of said cell in screening methods and methods

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

for diagnosing the genotype or the phenotype of said mutant ERalpha.

5. Claims: 1-14, 22, 30, 31, 39-55 all partially and 19, 27 completely

An artificial cell comprising i) a chromosome with a reporter gene, which comprises an ERE, a TATA sequence and a reporter sequence and ii) a mutant ERalpha having an amino acid sequence shown in SEQ.ID.7; an isolated mutant ERalpha having said sequence; a polynucleotide encoding said mutant ERalpha; use of said cell in screening methods and methods for diagnosing the genotype or the phenotype of said mutant ERalpha.

6. Claims: 1-14, 22, 30, 31, 39-55 all partially and 20, 28 completely

An artificial cell comprising i) a chromosome with a reporter gene, which comprises an ERE, a TATA sequence and a reporter sequence and ii) a mutant ERalpha having an amino acid sequence shown in SEQ.ID.9; an isolated mutant ERalpha having said sequence; a polynucleotide encoding said mutant ERalpha; use of said cell in screening methods and methods for diagnosing the genotype or the phenotype of said mutant ERalpha.

7. Claims: 1-14, 22 , 30, 31, 39-55 all partially and 21, 29 completely

An artificial cell comprising i) a chromosome with a reporter gene, which comprises an ERE, a TATA sequence and a reporter sequence and ii) a mutant ERalpha having an amino acid sequence shown in SEQ.ID.10; an isolated mutant ERalpha having said sequence; a polynucleotide encoding said mutant ERalpha; use of said cell in screening methods and methods for diagnosing the genotype or the phenotype of said mutant ERalpha.

8. Claims: 32-35

A method for quantitatively analysing transactivation activity

9. Claims: 36-38

a method for screening a test ligand dependent

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

transcriptional factor

NOTE: The non-unity is based on the fact that mutant ERalpha's having the phenotype as described in claim 12, as well as cells transfected with these mutants as described in claim 1, are already known in the prior art (see search report).

INTERNATIONAL SEARCH REPORT

International Application No. PCT/JP 00 /08553

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 48

Present claim 48 relates to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for NONE of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No
rcT/JP 00/08553

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8705049 A	27-08-1987	AU 7084787 A	09-09-1987
		EP 0258401 A	09-03-1988
		JP 63502397 T	14-09-1988